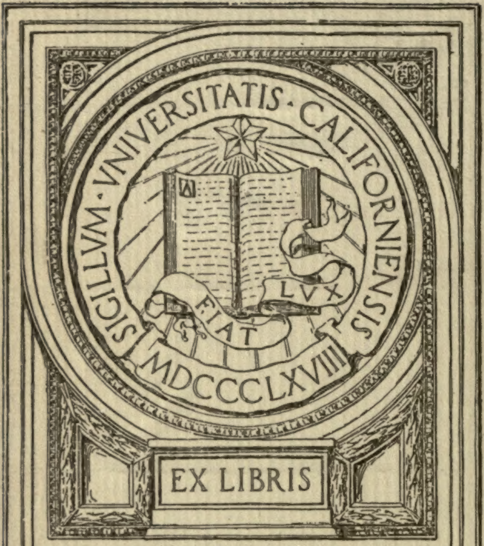

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Sodic chloride	0.700	7 gm
Calcic "	0.028	.23
Potassic "	0.030	.30

Worcester's Fluid.

10 % Formol } ?
Corr. Subl. to saturation.

ndicators –
2 gms Phenolphthalei
1 l 50% Alcohol
1 gm Methyl Orange to 1 l
distilled H_2O.

Litmus – Extract wit
H_2O, evap. to dryness, extrac
with 50% alcohol, dissol
residuum (not soluble in alc
in water.

THE

MICROTOMIST'S VADE-MECUM

First Edition	March, 1885.
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Sixth Do.	June, 1905.

THE

MICROTOMIST'S VADE-MECUM

mair.

A HANDBOOK OF THE METHODS OF MICROSCOPIC ANATOMY

BY

ARTHUR BOLLES LEE

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SIXTH EDITION

PHILADELPHIA
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PREFACE.

Although this new edition scarcely exceeds the last in length, it contains a great deal of new matter, as will appear at once by reference to the index, which contains some 300 new entries. Room for this added matter has been found partly by an even more severe condensation of the text than that which was carried out in the last edition, and partly by some rearrangements which have enabled me to avoid some unnecessary repetitions. The old chapter, "On Staining with Coal-tar Colours," has been suppressed, and its contents worked up into the chapter "Staining," which has been re-written so as to afford a more connected view of the whole subject. The chapters on the "Connective Tissues" and on "Blood and Glands" have been largely re-written, with much new matter. Particular attention has been paid to the chapters treating of the Nervous System. It will be remembered that in the Preface to the Fourth Edition it was explained that I was under great obligation to Professor van Gehuchten, who was so good as to revise these chapters for me, and to suggest a scheme for the arrangement of their contents. I have now very carefully rearranged them on the same lines, but effecting, as I think, some improvements in detail which conduce to a clearer view of the subject as a whole. One of these chapters—Chapter XXXII—has been so far re-written as to be practically new, and contains much new and important matter concerning the methods for Tigroid substance and Neurofibrils. The recent methods for Neuroglia have also received due attention.

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In order to be able to admit so much fresh matter without increasing the size of the book, it has been necessary throughout to suppress most carefully all unnecessary repetitions. All desirable explanations relating to the principles and theory of technical processes have therefore been relegated to the general chapters, such as those on "Killing," "Fixing," "Staining," "Imbedding," "Serial Section Mounting," "Impregnation Methods," and the like, and are not repeated in the special sections. These chapters must therefore be *read*—not treated as mere matter for occasional reference—in order to a due comprehension of the special sections and an intelligent utilisation of the book.

COLOGNY, GENEVA, SWITZERLAND;
June, 1905.

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THE MICROTOMIST'S VADE-MECUM.

CHAPTER I.

INTRODUCTORY.

1. The General Method.—The methods of modern microscopic anatomy may be roughly classed as General and Special. There is a General or Normal method which consists in carefully *fixing* the structures to be examined, *staining* them with a *nuclear* stain, *dehydrating* with alcohol, and mounting *series of sections* of the structures in *balsam.* It is by this method that the work is blocked out and very often finished. Special points are then studied, if necessary, by Special Methods, such as examination of the living tissue elements, *in situ* or in "indifferent" media; fixation with special fixing agents; staining with special stains; dissociation by teasing or maceration; injection; impregnation; and the like.

There is a further distinction which may be made, and which may help to simplify matters. The processes of the preparation of tissues may be divided into two stages, *Preliminary Preparation* and *Ulterior Preparation.* Now the processes of preliminary preparation are essentially identical in all the methods, essential divergences being only found in the details of ulterior preparation. By preliminary preparation is meant that group of processes whose object it is to get the tissues into a fit state for passing unharmed through all the ulterior processes to which it may be desired to submit them. It comprehends the operations of (1) killing; (2) fixing; (3) the washing and other manipulations necessary for removing the fixing agent from the tissues, and substituting for it the preservative liquid or other reagents which it is desired to employ. Ulterior preparation comprehends the processes sketched out in §§ 3 *et seq.*

2. Preliminary Preparation.—The first thing to be done with any structure is to *fix* its histological elements. (This statement applies equally to all classes of objects, whether it be desired to cut them into sections or to treat them in any other special way.) Two things are implied by the word "fixing": first, the rapid *killing* of the element, so that it may not have time to change the form it had during life, but is fixed in death in the attitude it normally had during life; and second, the *hardening* of it to such a degree as may enable it to resist without further change of form the action of the reagents with which it may subsequently be treated. Too much stress can hardly be laid on the importance of good fixation; without it it is impossible to get *good stains* or good sections, or preparations good in any way.

The structure having been duly fixed by one of the processes described in the chapter on Fixing Agents, is, except in special cases, *washed* in order to remove from the tissues as far as possible all traces of the fixing reagent.

The kind of liquid with which washing out is done is not a matter of indifference. If corrosive sublimate (for instance), or osmic acid, or a solution into which chromic acid or a chromate enters, have been used for fixing, the washing may be done with water. But if certain other agents, such as picric acid in any form, have been used, the washing must be done with alcohol. The reason of this difference is that the first-named reagents (and, indeed, all the compounds of the heavy metals used for fixing) enter into a state of chemical combination with the elements of tissues, rendering them insoluble in water; so that the hardening induced by these agents is not removed by subsequent treatment with water. Picric acid, on the other hand, produces only a very slight hardening of the tissues, so that the tissue elements are left in a soft state, in which they are obnoxious to all the hurtful effects of water. Alcohol must therefore be taken to remove the picric acid and to effect the necessary hardening at the same time. Instructions for washing out are given in the special sections.

These operations having been duly performed, two roads become open. The object may be further prepared by what may be termed the *wet* method, in which all subsequent operations are performed by means of aqueous media. Or it may be further prepared by the *dehydration* method, which consists in treatment with successive alcohols of gradually increasing strength, final *dehydration* with absolute alcohol, imbibition with an essential oil or other so-called *clearing* *agent* which serves to remove the alcohol, and lastly either mounting at once in balsam or other resinous medium or imbedding in paraffin for the purpose of making sections. The dehydration method is the course which is generally preferred, chiefly because of its great superiority as regards the preservation of tissues. The presence of water is the most important factor in the conditions that bring about the decomposition of organic matter, and its complete removal is the chief condition of permanent preservation. It is of course not intended here to suggest that wet methods of preparation should be altogether discarded. They have great value, they are even indispensable for special ends; and all that is intended to be suggested is that they should be regarded not as *general,* but as *special* methods.

3. Dehydration.—The further course of preparation by the dehydration method is as follows:—At the same time that the superfluous fixing agent is being removed from the tissues, or as soon as that is done, the *water of the tissues must be removed.* This is necessary for two reasons: first, in the interest of preservation, as above explained; and secondly, because all water must be removed in order to allow the tissues to be impregnated with the imbedding material necessary for section-cutting, or with the balsam with which they are to be finally preserved. This *dehydration* is performed as follows:—The objects are brought into weak alcohol, and are then passed through successive alcohols of gradually increased strength, remaining in each the time necessary for complete saturation, and the last bath consisting of absolute or at least very strong alcohol.

In dealing with extremely delicate objects, it may be necessary to take special precautions in order to avoid injury to them through the violent diffusion-currents that are set up in the passage from water to alcohol, or from one bath of alcohol to another of considerably different density. Some kind of diffusion-apparatus may conveniently be used in these cases. The objects may be placed with some of their liquid in a tube plugged at one end and closed at the other by a diaphragm of chamois skin or other suitable membrane, the tube being then immersed in a vessel containing the grade of alcohol that it is desired to add to the liquid in the tube, and the whole allowed to remain until by diffusion through the diaphragm the two liquids have become of equal density. Or, COBB's differentiator (*Proc. Linn. Soc.,* N.S.W., v, 1890,

p. 157; *Journ. Roy. Mic. Soc.*, 1890, p. 821) may be employed. This is a handy and efficient apparatus, and has, in contra-distinction to some similar ones, the advantage that it can be used to transfer from a heavy fluid to a lighter one. Or, the apparatus described and figured by HASWELL (*Proc. Linn. Soc.*, N.S.W., vi, 1891, p. 433; *Journ. Roy. Mic. Soc.*, 1892, p. 696). This consists of two wash-bottles connected in the usual way by tubing, and furnished, the one with an overflow-tube, and the other with a feeding-tube leading from an elevated reservoir connected with it by means of a regulating tap or drop arrangement. The objects are placed in the first bottle; some of the same liquid as that containing the objects is placed in the second bottle; and alcohol of the grade that it is desired to add is led into it from the reservoir. The mixture of liquids therefore takes place in the bottle that does not contain the objects, and the mixture itself is gradually led over to the objects through the siphon-tube connecting the two bottles. Another apparatus for rapid dehydration, devised by CHEATLE, will be found described in *Journ. Pathol. and Bacteriol.*, i, 1892, p. 253, or *Journ. Roy. Mic. Soc.*, 1892, p. 892. It is hardly simple enough to be recommendable. See also the apparatus of SCHULTZE (*Zeit. wiss. Mik.*, ii, 1885, p. 537), and that of KOLSTER (*ibid.*, xvii, 1900, p. 294).

I would here call attention to the varied usefulness of the "Siebdosen," or sieve-dishes of STEINACH, ZIMMERMANN, and SUCHANNEK (vide *Zeit. wiss. Mik.*, iv, 1887, p. 433, and vii, 1890, p. 158). They consist of a covered glass capsule into which is fitted a "sieve" made of a watch-glass pierced with holes and supported on legs, and are very handy, not only for staining, washing out, treatment with vapours, etc., but for any operation in which it is desirable to have specimens supported in the upper layers of a quantity of reagent. They are sent out in a very neat form by Grübler and Co. FAIRCHILD'S perforated porcelain cylinders for washing (*Zeit. wiss. Mik.*, xii, 1896, p. 301) seem to be a very neat idea. These are made small enough to be floated by the cork that closes them. See also the similar device of SCHAFFER (*ibid.*, xvi, 1900, p. 422; *Journ. Roy. Mic. Soc.*, 1900, p. 394). For EWALD'S section-washing apparatus, see *Zeit. Biol.*, xxxiv, 1897, p. 264.

A *capillary siphon* for the aspiration of liquids in the fixing, staining, and washing of suspended blood-corpuscles, sperm-cells, protozoa, and the like, is described by EWALD, *ibid.*, p. 253.

It is sometimes stated that it is necessary that the last alcohol-bath should consist of absolute alcohol. This, however, is incorrect, a strength of 90 per cent., or at all events 95 per cent., being sufficient in most cases. For the small amount of water that remains in the tissues after treatment with these grades of alcohol is efficiently removed in the bath of clearing agent if a good clearing agent be employed. Oil of cedar will remove the remaining water from tissues saturated with 95 per cent. alcohol; oil of bergamot will "clear" from 90 per cent. alcohol, and anilin oil will clear from 70 per cent. alcohol.

I am not aware of any substance that can entirely take the place of alcohol for dehydration and preservation. Acetone and methylal have been substituted for alcohol in the dehydration of methylen-blue preparations (PARKER, *Zool. Anz.*, 403, 1892, p. 376), and anilin oil can be made to dehydrate watery sections if they be first mopped up with blotting-paper; but a really efficient substitute for alcohol in general work remains yet to be discovered.

4. Preservation.—Considered as a mere dehydrating agent, *alcohol* fulfils its functions fairly well. But considered as a histological *preservative* agent, it is far less satisfactory. If tissues be left in alcohol for only a few days before further preparation, injurious effects will perhaps not be very disagreeably evident. But it is otherwise if they are put away in it for many weeks or months before the final preparation is carried out. The dehydrating action of the alcohol being continuously prolonged, the minute structure of tissues is sometimes considerably altered by it; they become over-hard and shrink, and become brittle, and their capacity for taking stains well becomes seriously diminished. KULTSCHITZKY (*Zeit. wiss. Mik.*, iv, 1887, p. 349) has proposed to remedy this by putting up objects, after fixation and washing out with alcohol, in ether, xylol, or toluol. FLEMMING (*Arch. mik. Anat.*, xxxvii, 1891, p. 685) advises putting up objects after fixation in a mixture of alcohol, glycerin, and water, in about equal parts, pointing out that objects thus preserved may be at any moment either prepared for sectioning by treatment with pure alcohol or softened for dissection or teasing by a little soaking in water, and that they do not become so hard and brittle as alcohol specimens, and retain their staining power much better. After extensive experience of this plan I can recommend it, and would only further suggest that the action of the liquid seems to me to be in many cases much improved by addition of a little acetic acid (say 0·5 to 0·75 per cent.).

For material that is intended *only for section-cutting*, I find that by far the best plan is to clear (next §) and imbed at once in paraffin. This affords, as far as I can see, an absolutely perfect preservation. Cedar-wood oil is, I find, nearly, if not quite, as good as paraffin, so far as the preservation of the tissues is concerned, but of course it is not so handy for storage.

5. Removal of Alcohol; Clearing.—The water having been sufficiently removed, as described in § 3, the alcohol is in its turn removed from the tissues, and its place taken by some anhydrous substance, generally an essential oil, which is miscible with the material used for imbedding or mounting. This operation is generally known as *Clearing*. It is very important that the passage from the last alcohol to the clearing agent be made *gradual*. This is effected by placing the clearing medium *under* the alcohol. A sufficient quantity of alcohol is placed in a tube (a watch-glass will do, but tubes are generally better), and then with a pipette a sufficient quantity of clearing medium is introduced *at the bottom of the alcohol*. Or you may first put the clearing medium into the tube, and then carefully pour the alcohol on to the top of it. The two fluids mingle but slowly. The objects to be cleared, being now quietly put into the supernatant alcohol, float at the surface of separation of the two fluids, the exchange of fluids takes place gradually, and the objects slowly sink down into the lower layer. When they have sunk to the bottom, the alcohol may be drawn off with a pipette, and after some further lapse of time the objects will be found to be completely penetrated by the clearing medium.

This method of making the passage from one fluid to another applies to all cases in which objects have to be transferred from a lighter to a denser fluid—for instance, from alcohol, or from water, to glycerin.

It should be noted here that this is the proper stage for carrying out *minute dissections*, if any such have to be done, a drop of clearing agent being a most helpful medium for carrying out such dissections (see § 9).

At this point the course of treatment follows one of two different roads, according as the object is to be mounted direct in balsam (§ 8), or is first to be sectioned (§ 6).

6. Imbedding, and Treatment of Sections.—The objects are now *imbedded*. They are removed from the clearing medium, and soaked until thoroughly saturated in the imbedding medium. This is, for small objects, generally paraffin, liquefied by heat, and for large objects generally a solution of collodion or "celloidin" (in this latter case the clearing may be omitted and the tissues be imbedded direct from the alcohol). The imbedding medium containing the object is then made to solidify, and sections are made with a microtome through the imbedding mass and the included objects. The sections are then mounted on a slide by one of the methods described in the chapter on Serial Section Methods, the imbedding material is removed from them (in the case of paraffin), they are stained *in situ* on the slide, dehydrated with alcohol, cleared, and mounted in balsam or damar. Or they may be stained, washed, dehydrated, and cleared in watch-glasses, and afterwards mounted as desired—the imbedding medium being first removed if desirable.

The plan of staining sections on the slide is of somewhat recent introduction; before it had been worked out the practice was to stain structures *in toto*, before cutting sections. In this case the object, after having been fixed and washed out, is taken from the water, or while still on its way through the lower alcohols (it should not be allowed to proceed to the higher grades of alcohol before staining, if that can be avoided), and passed through a bath of stain, then dehydrated with successive alcohols, passed through a clearing medium into paraffin, cut, and treated as above described, the sections in this case being mounted direct from the chloroform, xylol, or other solvent with which the paraffin is removed. If aqueous staining media be applied (and this is sometimes very desirable for particular purposes), the structures should either be stained *in toto* immediately after fixing and washing out, or sections may be stained on the slide, the objects, if delicate, being passed through successive baths of alcohol of gradually decreasing strength before being put into the aqueous stain.

In my opinion it is generally advisable not to stain in bulk material that is intended to be sectioned; by staining it as sections the staining can be much better controlled, and many excellent stains can in this way be employed that are not available for staining in bulk; and of course sections can be stained much more rapidly than material in bulk.

It may here be noted that balsam mounts of which the stain has faded, or which it may be desired to submit to some other staining process, or mount in some other medium, may often with great advantage be *re-stained* and *re-mounted*. All that is necessary is to put the slide into a tube of xylol or benzol till the cover falls off (about two days), wash well for some hours in clean xylol, and pass through alcohol into the new stain. Since this was pointed out to me by Dr. Henneguy I have unmounted and re-stained a large number of old preparations, and have succeeded in every case with series of sections mounted on Mayer's albumen, or by the water method. For shellac-mounted series, *see* E. MEYER, *Biol. Centralb.*, x, 1890, p. 509, or *last edition*.

The most convenient *vessels*, I find, in which to perform the various operations of staining, differentiating, dehydrating, clearing, etc., *on the slide*, are flat-bottomed corked glass tubes. I have mine made 10 centimetres high and 27 millimetres internal diameter. Each of these will then take two slides, English size, placed back to back. To make a stand for them, take a piece of deal board, 3 centimetres thick, and with a centrebit bore in it series of holes about 15 millimetres deep and of the diameter of the tubes, and about 3 centimetres apart lengthways and 1½ crossways. A board of 15 centimetres width and 45 length will take twenty-one tubes in three rows of seven each in the holes; and others may be stood up between the rows without much risk of their falling. I consider these tubes much more practical than the various racked troughs that have been recommended.

7. Résumé of the General Method.—To sum up, you may either fix, wash out, stain, wash, dehydrate, clear, imbed, cut sections, clear and mount them in balsam; or fix, wash, dehydrate, clear, imbed, cut, stain, wash, dehydrate, clear, and mount—according to choice.

8. Preparation of Entire Objects, or of Material that is not to be sectioned.—The treatment of objects which can be studied without being cut into sections is identical with that above described, with the omission of those passages that relate to imbedding processes. Its normal course may be described as fixation, washing out, staining, treatment with successive alcohols of gradually increasing strength, final dehydration with absolute alcohol, clearing, and mounting in balsam. This method is usually preferred, as a general method, to the wet methods, for the reasons that have been given above (§ 2), and for some others, amongst which may be noted the greater transparency given to tissues by mounting them in media of high refractive index, such as balsam.

In the *preparation of entire objects* or structures that are intact and *covered by an integument not easily permeable* by liquids, special care must be taken to avoid swelling from endosmosis on the passage of the objects from any of the liquids employed to a liquid of less density, or shrinkage from exosmosis on the passage to a liquid of greater density. This applies most specially to the passage from the last alcohol into the clearing medium. A slit should be made in the integument, if possible, so that the two fluids may mingle without hindrance. And in all cases the passage is made gradual by placing the clearing medium under the alcohol, as described (§ 5). Fluids of high diffusibility should be employed as far as possible in all the processes. Fixing agents of great penetrating power (such as picro-sulphuric acid or alcoholic sublimate solution) should be employed where the objects present a not easily permeable integument. Washing out is done with successive alcohols, water being used only in the case of fixation by osmic acid, or the chromic mixtures or other fixing solutions that render washing by water imperative. Staining is done by preference with alcoholic staining media. The stains most to be recommended are Grenacher's borax-carmine, or one of Mayer's alcoholic carminic acid or hæmatein stains (for all of which *see* STAINING AGENTS). Aqueous stains are more rarely indicated, though there are many cases in which they are admissible, and some in which they are preferable.

9. Minute Dissections.—These are best done, if necessary, in a drop of clearing agent. I recommend cedar-wood oil for this purpose, as it gives to the tissues a consistency very favourable for dissection, whilst its viscosity serves to lend support to delicate structures. Clove oil has a tendency to make tissues that have lain in it for some time very brittle. The brittleness is, however, sometimes very helpful in minute dissections. Another property of clove oil is that it does not easily spread itself over the surface of a slide, but has a tendency to form very convex drops, and this also makes it frequently a very convenient medium for making minute dissections in.

If it be desired to dissect in a watery fluid, such as glycerin, it may be well to prepare the slide by spreading on it a thin layer of Mayer's albumen (§ 201), and on this place a small drop of glycerin, or other dissecting medium. As soon as the dissection has been accomplished, a cover may be let fall, horizontally, on to the preparation, and a weight placed on it. Then the mount may be filled up with glycerin, or other mounting medium, run in under the cover, and closed, if desired (Mayer, *Grundzüge*, p. 10).

CHAPTER II.

KILLING.

10. In the majority of cases, the first step in the preparation of an organ or organism consists in exposing it as rapidly and as completely as possible to the action of one of the **Fixing Agents** that are discussed in the next chapter. The organ or organism is thus taken in the normal living state; the fixing agent serves to bring about at the same time, and with sufficient rapidity, both the death of the organism and that of its histological elements.

But this method is by no means applicable to all cases. There are many animals, especially such as are of a soft consistence, and deprived of any rigid skeleton, but possessing a considerable faculty of contractility—such as many Cœlenterata, Bryozoa, and Serpulida, for instance—which if thus treated contract violently, draw in their tentacles or branchiæ, and die in a state of contraction that renders the preserved object a mere caricature of the living animal. In these cases special methods of killing must be resorted to.

Sudden Killing.

11. Heat.—Speaking generally, there are two ways of dealing with these difficult cases. You may kill the animal so suddenly that it has not time to contract: or you may paralyse it by narcotics before killing it.

The application of *Heat* affords a means of killing suddenly. It has the advantage of allowing of good staining subsequently, and of hindering less than any other method the application of chemical tests to the tissues. By it the tissues are fixed at the same time that somatic death is brought about.

The difficulty consists in hitting off the right temperature, which is of course different for different objects. I think that a temperature of 80° to 90° C. will generally be amply sufficient, and that very frequently it will not be necessary to go beyond 60° C. An exposure to heat for a few seconds will generally suffice.

Small objects (Protozoa, Hydroids, Bryozoa) may be brought into a drop of water in a watch-glass or on a slide, and heated over the flame of a spirit-lamp. For large objects, the water or other liquid employed as the vehicle of the heat may be heated beforehand and the animals thrown into it.

As soon as it is supposed that the protoplasm of the tissues is coagulated throughout, the animals should be brought into alcohol (30 to 70 per cent. alcohol) (if water be employed as the heating agent).

An excellent plan for preparing many marine animals is to kill them in *hot fresh* water. Some of the larger Nemertians are better preserved by this method than by any other with which I am acquainted.

See also §§ 638 to 644.

12. Slowly Contracting Animals.—Animals that contract but slowly, such as *Alcyonium* and *Veretillum*, and some Tunicates, such as *Pyrosoma*, are very well killed by throwing them into some very quickly acting fixing liquid, either used hot or cold. *Glacial* or very strong *acetic acid* (VAN BENEDEN'S method) is an excellent reagent for this purpose; it may be used, for example, with some Medusæ. After an immersion of a few seconds or a few minutes, according to the size of the animals, they should be brought into alcohol of at least 50 per cent. strength. See "**Acetic acid**" and "**Tunicata.**" Lemon juice employed in this way has given me very good results with small Annelids and Hirudinea. *Corrosive sublimate* is another excellent reagent for this purpose.

Narcotisation.

13. The secret of narcotisation consists in adding some anæsthetic substance very gradually, in very small doses, to the water containing the animals, and waiting patiently for it to take effect slowly.

The Tobacco-smoke Method for Actiniæ, due to LO BIANCO (*Jena Zeit. Naturw.*, Bd. xiii, 1879, p. 467; *Mitth. Zool. Stat. Neapel*, Bd. ix, 1890, p. 499), used to be practised as follows:—A dish containing the animals in water is covered with a bell-glass, under which passes a curved glass or rubber tube, which dips into the water. Tobacco smoke is blown into the water for some time through the tube, and the animals are then left for some hours. More smoke is then blown in, and the animals are left overnight. Next morning they should be irritated from time to time by touching a tentacle with a needle. As soon as it is observed that the contraction of the tentacle does not begin until a considerable time after it has been irritated by the needle, the narcotisation may be considered sufficient. A quantity of some fixing liquid sufficient to kill the animals before they have time to contract is then added to the water.

14. Nicotin in solution may be used instead of tobacco smoke (Andres, *Atti R. Accad. dei Lincei*, v, 1880, p. 9). Andres employs a solution of 1 gramme of nicotin in a litre of sea water. The animal to be anæsthetised is placed in a jar containing half a litre of sea water, and the solution of nicotin is gradually conducted into the jar by means of a thread acting as a siphon. The thread ought to be of such a thickness as to be capable of carrying over the whole of the solution of nicotin in twenty-four hours. See also *Mitth. Zool. Stat. Neapel*, Bd. ii, 1880, p. 123.

15. Chloroform may be employed either in the liquid state or in the state of vapour. Korotneff (*Mitth. Zool. Stat. Neapel*, v, 1884, p. 233) operates in the following manner with Siphonophora. The animals being extended, a watch-glass containing chloroform is floated on the surface of the water in which they are contained, and the whole is covered with a bell-glass. As soon as the animals have become insensible they are killed by means of hot sublimate or chromic acid solution plentifully poured on to them.

Liquid chloroform is employed by squirting it in small quantities on to the surface of the water containing the animals. A syringe or pipette having a very small orifice, so as to thoroughly pulverise the chloroform, should be employed. Small quantities only should be projected at a time, and the dose should be repeated every five minutes until the animals are anæsthetised.

I have seen large Medusæ very completely anæsthetised in the state of extension in an hour or two by this method. Andres finds that this plan does not succeed with Actiniæ, as with them maceration of the tissues supervenes before anæsthesia is established.

Preyer (*Mitt. Zool. Stat. Neapel*, Bd. vii, 1886, p. 27) recommends chloroform water for star-fishes.

16. **Ether** and **Alcohol** may be administered in the same way. Andres has obtained good results with Actiniæ by the use of a mixture (invented by Salvatore lo Bianco) containing 20 parts of glycerine, 40 parts of 70 per cent. alcohol, and 40 parts of sea water. This mixture should be carefully poured on to the surface of the water containing the animals, and allowed to diffuse quietly through it. Several hours are sometimes necessary for this.

Eisig (*Fauna u. Flora Golf. Neapel*, 16, 1887, p. 239) benumbs Capitellidæ by putting them into a mixture of one part of 70 per cent. alcohol with 9 parts of sea water.

Oestergren (*Zeit. wiss. Mik.*, xix, 1903, p. 300) makes a saturated (7 to 8 per cent.) solution of ether in sea or soft water, and uses it either concentrated or diluted to about 1 per cent., and finds it to succeed with all classes of aquatic animals.

17. **Methyl-alcohol.**—Cori (*Zeit. wiss. Mik.*, vi, 1890, p. 438). Cori recommends a mixture composed of 10 c.c. methyl-alcohol (of 96 per cent. strength), 90 c.c. water (fresh or sea water), and 0·6 grm. of sodium chloride (to be added only when fresh water is taken, the addition of the salt having for its object to prevent maceration). It may be well to add to this mixture a very few drops of chloroform (for *Cristatella; Zeit. wiss Zool.*, lv, 1893, p. 626).

18. **Hydrate of Chloral**, which was first recommended, I believe, by Foettinger (*Arch. de Biol.*, vi, 1885, p. 115), gives very good results with some subjects. Foettinger operates by dropping crystals of chloral into the water containing the animals. For *Alcyonella* he takes 25 to 80 centigrammes of chloral for each hundred grammes of water. It takes about three quarters of an hour to render a colony sufficiently insensible to allow of fixing. Foettinger has obtained satisfactory results with marine and fresh-water Bryozoa, with Annelida.

Mollusca, Nemertians, Actiniæ, and with *Asteracanthion*. He did not succeed with Hydroids.

Lo Bianco (*Mitth. Zool. Stat. Neapel*, Bd. ix, 1890, p. 442) employs for various marine animals freshly prepared solutions of chloral in sea water, of from one tenth to one fifth per cent. strength.

I am bound to state that I have never had the slightest success with Nemertians.

Verworn (*Zeit. wiss. Zool.*, xlvi, 1887, p. 99) puts *Cristatella* for a few minutes into 10 per cent. solution of chloral, in which the animals sooner or later become extended.

Kükenthal (*Jena Zeit. Naturw.*, Bd. xx, 1887, p. 511) has obtained good results with some Annelids by means of a solution of one part of chloral in 1000 parts of sea water.

The chloral method gives rise to maceration with some subjects, as I can testify, and has been said to distort nuclear figures.

19. Cocaine (Richards, *Zool. Anz.*, 196, 1885, p. 332). Richards puts a colony of Bryozoa into a watch-glass with 5 c.c. of water, and adds gradually 1 per cent. solution of hydrochlorate of cocaine in water. After five minutes the animals are somewhat numbed, and half a cubic centimetre of the solution is added; and ten minutes later the animals should be found to be dead in a state of extension.

This method is stated to succeed with Bryozoa, *Hydra*, and certain worms. It is the best method for Rotifers (Rousselet). It has also been recommended for *Aplysia*.

It has been pointed out (by Cori, in the paper quoted § 17) that, unfortunately, when fixing agents, such as sublimate solution, are added to the animals, the cocaine is thrown down on them as a white precipitate. This precipitate, however, may be redissolved afterwards in alcohol (Eisig).

Cocaine solutions cannot be depended on to keep for more than a few days.

19a. Eucain. Harris (*Journ. Roy. Mic. Soc.*, 1900, p. 404) recommends a 1 per cent. solution of eucain hydrochloride, as giving far better results, with Vorticellidæ, Rotatoria, and Vermes. Rousselet (*ibid.*) reports favourably as to its action on Flosculariæ. It is stated to be perfectly stable in aqueous media.

20. Hydroxylamin.—Hofer (*Zeit. wiss. Mik.*, vii, 1890, p. 318) has employed hydroxylamin. Either the sulphate or, preferably, the hydrochlorate of the base may be used. This, as found in commerce, is usually contaminated with HCl. It should be dissolved in water (spring or sea water, according to the habitat of the organisms—in no case distilled water), and the solution exactly neutralised by addition of carbonate of soda. The organisms are placed in a solution diluted to about 0·1 per cent., used for thirty minutes or less (as for Infusoria), to 0·25 per cent., used for from fifteen minutes to one hour (*Hydra*), 1 per cent., one half to two hours (*Hirudo*), or as much as ten to twenty hours (*Helix* and *Anodonta*).

Hydroxylamin is a powerful reducing agent. Care must therefore be taken not to treat the paralysed animals with easily reducible fixing agents, such as osmic acid, chromic acid, sublimate, chlorides of gold or platinum, etc., unless it has been possible first to sufficiently wash out the hydroxylamin with water.

21. Chloride or Sulphate of Magnesium.—Tullberg (*Arch. Zool. Expér. et Gén.*, x, 1892, p. 11) has obtained some results with these salts. For Actiniæ, a 33 per cent. solution of the chloride is to be very slowly added to the water containing the expanded animal, until the vessel contains 1 per cent. of the salt (thus for one litre of sea water 33 c.c. of the solution must be added). The addition must be made gradually, but it must be effected within half an hour. Thirty minutes later the animal will be found to be anæsthetised, and may be fixed.

For terrestrial and fresh-water Invertebrates rather stronger solutions should be used.

Redenbaugh (*Amer. Natural*, xxix, 1895, p. 399) has obtained good results by means of the sulphate, either added in crystals to the sea water containing the animals until a saturated solution is obtained, or in the shape of a saturated solution into which they are thrown (Annelids).

22. Poisoning by small doses of some fixing agent is sometimes a good method. Salvatore lo Bianco kills *Ascidia* and *Rhopalæa* in an extended state (*Mitth. Zool. Stat. Neapel*, ix, 1890, p. 471) by pouring a little 1 per cent. chromic acid on to the surface of the water containing the animals, and allowing it to slowly diffuse into it. About twelve to twenty-four hours is necessary. He kills *Ciona* in a similar way with a mixture of one part of 1 per cent. chromic acid and nine parts of 49 per cent. acetic acid.

Osmic acid, or Kleinenberg's solution, is sometimes employed in the same way.

I have seen Medusæ killed in a satisfactory manner by means of crystals of corrosive sublimate added to the water containing them.

Morphia, Curare, Strychnin, Prussic Acid, and other paralysing drugs, have also been employed.

23. **Asphyxiation** may be sometimes successfully practised. Terrestrial Gastropods may be killed for dissection by putting them into a jar quite full of water that has been deprived of its air by boiling, and hermetically closed. After from twelve to twenty-four hours the animals are generally found dead and extended. The effect is obtained somewhat quicker if a little tobacco be added to the water.

Good results are sometimes obtained with aquatic animals by simply leaving them to exhaust the oxygen of the water in which they are contained. I have sometimes succeeded with Holothuriæ and other Echinoderms in this way. Ward (see *Amer. Nat.*, xxv, 1891, p. 398) has succeeded with Hydroids, Actiniæ, and similar forms, and Uexküll (*Mitth. Zool. Stat. Neapel*, xii, 1896, p. 463) with Echinids. If the animals be found to be imperfectly expanded when narcosis has set in, they may be got to expand by putting them back for a short time into pure sea water; and as soon as they are expanded should be quickly thrown into some rapidly killing reagent.

Marine Animals are sometimes successfully killed by simply putting them into fresh water.

Warm Water will sometimes serve to immobilise and even kill both marine and fresh-water organisms.

24. **Carbonic Acid Gas** has been recommended (by Fol, *Zool. Anz.*, 128, 1885, p. 698). The water containing the animals should be saturated with the gas. The method is stated to succeed with most Cœlenterata and Echinodermata, but not with Molluscs or Fishes. I have had most excellent results with small Annelids and Hirudinea. It is not necessary to employ a generator for obtaining the gas. It suffices to take an ordinary "soda-water" siphon, and squirt its contents into the water containing the animals.

Narcotisation is very rapidly obtained with very small animals, but much more slowly with larger ones. For instance, *Stylaria proboscidea*, I find, is paralysed in a few *seconds*; a small *Nephelis*, of 15 or 20 millimetres in length, will require about five *minutes*; and a large Nephelis, of from 10 to 15 centimetres, will require as many *hours*. Narcotised animals recover very quickly on being put back into pure water.

Uexküll (*Mitth. Zool. Stat. Neapel*, xii, 1896, p. 463) has paralysed Echinids very rapidly with carbonic acid, likewise a small Teleostean fish; whilst *Scyllium* and Crustaceans were affected much more slowly, and mussels not at all.

25. Peroxide of Hydrogen.—Volk (*Zool. Anz.*, xix, 1896, p. 294) kills Rotatoria by means of one or two drops of a 3 per cent. solution added to 1 c.c. of the water containing them. They die extended, and are then brought first into pure water and then into some fixing liquid.

CHAPTER III.

FIXING AND HARDENING.

26. The Functions of Fixing Agents.—The meaning of the term "fixing" has been explained above (§ 2). It remains here to insist on the absolute necessity of the employment of fixing agents, and to briefly illustrate this necessity. If a portion of living retina be placed in aqueous humour, serum, or other so-called "indifferent" medium, or in any of the media used for permanent preservation, it will be found that the rods and cones will not preserve the appearance they have during life for more than a very short time; after a few minutes a series of changes begins to take place, by which the outer segments of both rods and cones become split into discs, and finally disintegrate so as to be altogether unrecognisable, even if not totally destroyed. Further, in an equally short time the nerve-fibres become varicose, and appear to be thickly studded with spindle-shaped knots; and other post-mortem changes rapidly occur. If, however, a fresh piece of retina be treated with a strong solution of osmic acid, the whole of the rods and cones will be found perfectly preserved after twenty-four hours' time, and the nerve-fibres will be found not to be varicose. After this preliminary hardening, portions of the retina may be treated with water (which would be ruinous to the structures of a fresh retina), they may even remain in water for days without harm; they may be stained, acidified, hardened, imbedded, cut into sections, and mounted in either aqueous or resinous media without suffering.

This example shows that one of the objects aimed at in fixing is to impart to tissues the degree of *hardening* necessary to enable them to offer such mechanical resistance to *post-mortem* change and to the processes of after-treatment as not to suffer change of form. Another important function of fixing is to *render insoluble* elements of cells and tissues that would otherwise be more or less dissolved out by the liquids employed during the after-treatment. Compare in this respect the aspect of sections of a piece of testis that has been well fixed in liquid of Flemming and cut in paraffin, with the aspect of paraffin sections of a piece of the same testis that has not been fixed, or that has only been fixed by some reagent inadequate for the purpose, such as alcohol or picric acid. In the one case, plump, full, unshrunken cells, free from vacuoles, full of structure ; in the other, lean, empty, shrunken cells, with foamy and vacuolated protoplasm, half their original structure lost, and that which remains distorted ! Their appearance, compared with that of living or well-preserved cells, suggests at once that much must have been dissolved out of them.

A third and highly important function of fixing agents consists in producing *optical differentiation* in structures. By coagulating the elements of tissues and cells, fixing agents *alter their indices of refraction,* raising them in varying degrees. They do not act in an equal degree on all the constituent elements of cells and tissues, but raise the index of some more than that of others, thus producing optical differentiation where there was little or none before. Compare the aspect of the epithelium of the tail of a living tadpole, observed in water, with its aspect after the action of a little diluted solution of Flemming. In the living state the protoplasm of its cells has a refractive index little superior to that of water, and consequently so low an index of visibility that hardly any structure can be made out in the object. But as soon as the protoplasm has been sufficiently coagulated by the reagent the refractive indices of some of its elements will have been raised to above that of balsam, the chromatin of the nuclei will be brought out, and other structures be revealed where none was visible before.

The notion of fixing is distinct from that of *hardening.* All fixing presupposes a certain degree of hardening, as explained above. But it does not include the degree of hardening necessary to give to soft tissues a consistency which will allow them to be cut into thin sections without imbedding. *This* is hardening proper (see § 31). Of course, if the stage of fixing be prolonged, with a view to procuring enhanced optical differentiation, hardening will be superinduced, and the one stage will run into the other.

27. The Action of Fixing Agents consists in coagulating and rendering insoluble certain of the constituents of tissues. This is effected sometimes without any chemical action being involved, as when alcohol is employed, which acts by simple withdrawal of the water of the tissues. But in the majority of cases the fixing agents enter into chemical combination with certain of the elements of the tissues. The compounds thus formed are sometimes unstable and soluble, so that they are removable by washing, as is the case with several of those formed by picric acid. It is found in practice, however, that those formed by chromic acid and its salts, and the salts of the heavy metals, as mercury, iron, platinum, gold, and silver, are mostly insoluble.

The insolubility of these bodies is an advantage from the point of view explained in the last section, in that it ensures that the tissues shall not be robbed of their essential constituents, nor deprived of their desired consistency and optical differentiation, by the reagents subsequently employed. It is also sometimes an advantage in that certain of the compounds in question have the property of combining with certain colouring matters, and thus affording important stains which could not otherwise be obtained; or in other words, of acting as mordants.

But it is sometimes a disadvantage, inasmuch as these same compounds which render possible the production of some stains are hindrances to the production of others. Tissues that have been fixed with osmic or chromic acid or its salts are in general not easily to be stained with carmine or similar colouring matters, unless the metals have been previously removed by special chemical treatment (see § 41, and BLEACHING); though they may generally be stained with hæmalum, or, after sectioning, with iron hæmatoxylin or tar colours. According to FISCHER (*Fixirung, Färbung, und Bau des Protoplasmas*, Jena, G. Fischer, 1899), the coagulation which constitutes fixation is, in the case of the liquid and semi-liquid constituents of tissues, *always* a phenomenon of *precipitation*. The more solid constituents (such as fibrils that are visible during life, nucleoli, and the like) he admits may be acted on by fixing reagents without the formation of any visible precipitates. But all the liquid ones, in so far as they are fixed at all, are visibly precipitated in special *precipitation forms*, which vary according to the precipitant. Each fixing agent gives its own characteristic *fixation image*, which may be more or less lifelike, but can never be absolutely so. Fischer gives copious descriptions of the precipitation forms of the chief organic compounds found in tissues, and of the precipitation powers of the chief fixing agents, which the reader will do well to study.

It seems to be in consequence of Fischer's theory of fixation by precipitation that the most energetic fixing agents should always be found amongst the most energetic precipitants. But on the showing of his experiments this is not so. For instance, it is allowed on all hands that osmic acid is a most energetic fixative. But Fischer finds (*op. cit.*, pp. 12—14, 27) that it is a very incomplete and weak precipitant. Or, to take a contrary instance, he finds that picric acid is an energetic precipitant of the majority of cell constituents; but surely every cytologist must admit that it is a very incomplete fixative.

It would seem to follow, from these instances and from other similar ones, that Fischer's tables of precipitating power cannot be taken as a measure of the fixing power of the reagents. And further, the study of the fixation images of tissues afforded by osmic acid, formaldehyde, and other reagents, seems to show that the coagulation brought about by them is in part accompanied by the formation of precipitates, but in part not so, and that they may do their work to a larger extent than he seems to admit through a *homogeneous coagulation*. But from his very suggestive observations and reasonings it certainly appears that the formation of visible precipitates is a very wide-spread, if not universal concomitant of fixation; and that the wider the precipitating power of a fixative (*i.e.* the greater the number of organic liquids that it can precipitate), the greater will be the *number of artefacts* to which it can give rise. His work is deserving of most careful study.

28. The Characters of the Usual Fixing Agents.—A good fixing agent should first of all *preserve* all the elements it is desired to fix. But that is not enough; it should also give good optical *differentiation*, and should have sufficient power of *penetration* to ensure that small pieces of tissue be equally fixed by it throughout. A perfect fixing agent should also be such as not to interfere in any way with subsequent staining. Now no single substance or chemical compound fufils all that is required of a good fixing agent; hence it is that without exception all the best fixing agents are *mixtures*. Osmic acid, for instance, fulfils some of these conditions, but not all of them. It kills rapidly, and preserves admirably the elements of cytoplasm, but nuclei not so well. But the optical differentiation that it gives, though sometimes good, is often very inferior. For osmic acid, by coagulating in nearly equal degrees alike the spongioplasm (the plastin reticulum) and the hyaloplasm (the enchylema) of the cell-body, and the chromatin of nuclei, raises alike the refractive indices of all of them; so that if the fixing action have been in the least degree overdone, the cells acquire a homogeneous aspect in which the finer details are obscured by the general refractivity of the whole. If now, instead of using it pure, it be used in combination with acetic acid, a better differentiation is obtained; for acetic acid is properly a fixative only for a limited time, whilst, if its operation be prolonged, it exercises a swelling and solvent action on the elements of protoplasm. It therefore, whilst enhancing, or at all events not interfering with the fixation of the chromatin, serves to facilitate penetration and to counteract the excessive action of the osmic acid on the protoplasm, so that the cells come out less homogeneous and with more detail observable in them. A still better effect is obtained if to the osmic acid there be added not only acetic acid, but also chromic acid. For osmic acid has the property of blackening tissues, thus rendering them opaque. Chromic acid counteracts in a considerable degree this blackening action. It also helps, probably, to bring out the chromatin of nuclei, which is insufficiently fixed by the other two ingredients, and probably also helps in other ways to bring about optical differentiation; so that in the result a much clearer picture is obtained.

Such a mixture gives admirable results so far as preservation and differentiation are concerned. But as regards *penetration* it gives only very bad results. Osmic acid is hopelessly deficient in power of penetration, and no admixture with other substances has been successful in curing this defect. So that whenever a fair degree of penetration is required, some other reagent must be resorted to. Picric acid is one of the most penetrating fixatives known, but its hardening power is very slight, so that in order to produce the best results it ought to be combined with some more energetically hardening reagent. Corrosive sublimate is very penetrating and hardens very energetically, but in no form in which I have tried it does it give the fine optical differentiations that are obtained by means of the osmic mixtures. Bichromate of potash is an admirable preservative of protoplasm, but is not very penetrating, and does not properly preserve the chromatin of nuclei, causing it to swell. This defect may be overcome by combining it with sufficient acetic acid; but the defect of want of penetration will still remain.

I take it that it has been established by experience that, as a general rule, in order to get the best results, all fixatives should have an *acid reaction*. Consequently, if their chief ingredients have not naturally an acid reaction, they should be acidified, *e.g.* osmic acid should be acidified with acetic acid or the like. As a matter of fact, it will be found that acetic acid is very largely employed in mixtures. It is generally held that it acts in them as an agent for facilitating penetration and producing differentiation, as explained above, and also for ensuring the fixation of nuclein (if the other ingredients are not adequate thereto,—as, for example, in the case of bichromate of potash); but this is probably not all. Fischer (in the work quoted § 27, pp. 10, 27, and other places) holds that its function in these mixtures is chiefly that of a *neutraliser* or *acidifier* (Ansäurer) for ensuring that the other ingredients shall have an acid, or at least a neutral medium to do their work in. For the precipitating power, that is, in his view, the fixing power of a reagent, varies according to the reaction, acid or alkaline, of the things to be fixed; and a weakly acid reaction is the one most favourable for ensuring precipitation. Many things that are quite unprecipitable by certain reagents whilst in alkaline or neutral solution are immediately precipitated by them if the solution is rendered acid. "Many kinds of cell contents," he says (*op. cit.*, p. 10) "indeed the majority, have an alkaline reaction, and are thereby quite inaccessible to the precipitating action of certain agents, such as osmic acid, or bichromate; and the action of certain other fixatives, such as platinum chloride and chromic acid, is more or less hindered by the presence of free alkalies. For neither the chromic acid (of solution of Flemming) nor the platinum chloride (of solution of Hermann) would be adequate to act as acidifiers to the osmic acid of the mixtures. They cannot do so, first, because they themselves become combined with the tissues much more quickly than the slowly working osmic acid, and secondly, because they themselves have only an extremely weak acid reaction." Hence the function of the organic acid is to bring into play the precipitating power of the other ingredients.

Not only is it true that the most complete fixations can only be obtained by means of mixtures, but it is also true that no one mixture can serve all ends. It is probably misleading to recommend this or that reagent as "the best for general purposes," or the like. It may, for instance, often be advisable to refrain from using an otherwise suitable fixative on account of its unfavourable influence on subsequent staining. And as regards the mere fixation itself, it is undoubtedly the case that, to ensure the best results, *different objects require different fixations*:—some of them quite special ones.

Speaking generally, osmic acid, chromic acid, bichromates, chloride of platinum, and the majority of the compounds of the heavy metals, are *hindrances* to staining; whilst heat, alcohol, trichloracetic acid, formol, corrosive sublimate, nitric acid, picric acid, and acetic acid, are *neutral*, or even *favourable*, in this respect.

I think *the beginner* should avoid such things as liquid of FLEMMING and similar mixtures. He may take, instead, liquid of TELLYESNICZKY. This gives a fair fixation, and is easy to manage, but it is wanting in penetration.

Corrosive sublimate is a good all-round fixative, with excellent penetration, but is not quite so easy to manage.

Picric acid gives a fair though weak fixation, with very good penetration, is easy to manage, and does not make tissues brittle, which sublimate easily may do. Combined with formol, as in the liquid of BOUIN, § 117, it gives most excellent results.

To ensure the best results, *all fixatives should be acid*, for the reasons explained above. They may conveniently be made to contain from one to five per cent. of acetic acid.

See, further, besides the work of FISCHER quoted § 27, TELLYESNICZKY, *Arch. mik. Anat.*, lii, 2, 1898; WASIELEWSKI, *Zeit. wiss. mik.*, xvi, 1899; and BERG, *Arch. f. Mik. Anat.*, lxii, 1903, p. 367.

29. The Practice of Fixation.—Hints and Cautions.—See that the structures are *perfectly living* at the instant of fixation, otherwise you will only fix pathological states or post-mortem states.

Fixation is generally performed by *immersion* of the objects in the fixing liquid. In this case, everything should be done to facilitate the *rapid penetration* of the fixing agent. To this end let the structures be divided into the smallest portions that can conveniently be employed, and if entire organs or organisms are to be fixed whole, let openings, as large as possible, be first made in them.

The penetration of reagents is greatly facilitated by *heat*. You may warm the reagent and put it with the object to be fixed in the paraffin stove, or you may even employ a fixing agent heated to boiling-point (as boiling sublimate solution for certain corals and Hydroids, or boiling absolute alcohol for certain Arthropods with very resistent integuments). But this should only be done as a last resource.

Let the *quantity* of fixing agent employed be at least *many times* the volume of the objects to be fixed. If this precaution be not observed the composition of the fixing liquid may be seriously altered by admixture of the liquids or of the soluble substances of the tissues thrown into it. For a weak and slowly acting fixing agent, such as picric acid, the quantity of liquid employed should be in volume about one hundred times that of the object to be fixed. Reagents that act very energetically, such as Flemming's solution, may be employed in smaller proportions.

But fixation may also be performed by *injection* of the fixing liquid into the objects, thus ensuring a more rapid and thorough penetration of *voluminous* objects by the reagents than can be obtained by simple immersion. See for this practice the methods of fixation by injection of GOLGI, DE QUERVAIN, and others, given under *Neurological Methods* (section methods).

Braus and **Druener** (*Jena. Zeit. Naturw.*, Bd. xxix, 1895, p. 435) fix fishes *by injection* through the *bulbus aortæ*. The vessels are first washed out with normal salt solution, the fixing liquid is then thrown in, then, as soon as fixation is judged to be complete, water is injected; lastly, alcohol, and the fishes are thrown into alcohol. Or, if chromic liquids have been employed, the fishes are put direct into solution of Müller.

As regards the *time* during which fixing reagents should be allowed to act, it is well, as a general rule, not to leave specimens in them for a longer time than is sufficient to obtain the desired reaction. Sublimate, for instance, soon makes tissues brittle. But long immersion may be necessary to produce the desired optical differentiation with some reagents.

Careful *washing out* (by which is meant the removal from the tissues of the excess of uncombined fixative) is necessary in order to get tissues to stain properly. But it is not always equally imperative. Alcohol and formaldehyde do not require washing out before staining; acetic and picric acid only for some stains; sublimate will allow of staining even if not washed out, but allows of a sharper stain if well washed out; all osmic, chromic, and platinic liquids require very thorough washing out.

Be careful to use the *appropriate liquid for washing out* the fixing agent after fixation. It is frequently by no means a matter of indifference whether water or alcohol be employed for washing out. Sometimes water will undo the whole work of fixation (as with picric acid). Sometimes alcohol causes precipitates that may ruin the preparations. Objects fixed in alcohol, formol, acetic acid, picric acid, or nitric acid require to be washed out with alcohol, or at least with some hardening liquid, whilst those that have been fixed with osmic or chromic acid, or with one of the other compounds of the heavy metals, require *in general* to be washed out with water. Sublimate, however, is best washed out with alcohol. Instructions on this head are given where necessary.

Use *liberal quantities* of liquid for washing.

Change the liquid as often as it becomes turbid, if that should happen.

The process of washing out is often greatly facilitated by *heat*. Picric acid, for instance, is nearly twice as soluble in alcohol warmed to 40° C. as in alcohol at the normal temperature (Fol).

30. Fixation of Marine Animals.—In the case of *marine organisms* it may be stated as a general rule that their tissues are more refractory to the action of reagents than are the tissues of corresponding fresh-water or terrestrial forms, and fixing solutions should in consequence be stronger (about two to three times stronger, according to Langerhans).

Marine animals ought to be *freed from the sea water* adherent to their surface before treating them either with alcohol or any fixing reagent that precipitates the salts of sea water. If this be not done, the precipitated salts will form on the surfaces of the organisms a crust that prevents the penetration of reagents to the interior, thus allowing maceration to be set up, and hindering the penetration of staining fluids. Fixing solutions for marine organisms should therefore be such as serve to keep in a state of solution, and finally remove, the salts in question. As a general rule, they should *not be made with sea water* as a menstruum. If, however, for any particular purpose, it is deemed desirable so to prepare them, care should be taken to remove the sea salts afterwards by appropriate washing, or to put up the objects in glycerin (MAYER). If alcohol be employed, it should be *acidified* with hydrochloric or some other appropriate acid. Picro-nitric acid is a fixing reagent that fulfils the conditions here spoken of. (On this subject see MAYER, in *Mitth. Zool. Stat. Neapel*, ii (1881), p. 1, *et seq.*)

31. Hardening.—The process of hardening was above (§ 26) distinguished from that of fixing as being directed to the attainment of a degree of consistency sufficient to allow of soft tissues being cut into sections without imbedding. It is also distinguished from fixing in that it does not include the killing of the elements. Nerve tissue, for instance, is daily hardened after having come into the hands of the anatomist some twelve or twenty-four hours after the death of the subject, under which conditions there can, of course, be no question of fixing. Hardening is an *after-process,* and only ranks as a *special* method.

Methods of imbedding have now been brought to such a degree of perfection that the thorough hardening of soft tissues that was formerly necessary in order to cut thin sections from them is, in the majority of cases, no longer necessary. But there are some exceptions. Such are, for instance, the cases in which it is desired to cut very large sections, such as sections of the entire human brain. Such an organ as this cannot be duly infiltrated with paraffin or any other imbedding mass without inordinate expenditure of time. And certain organs that are either extremely delicate or inaccessible, such as retina or cochlea, will require to be specially hardened in order to give the best results. For these, see *Neurological Methods*.

The reagents employed for hardening are for the most part of the same nature as those employed for fixing. But it does not follow that all fixing agents can be employed for hardening. Corrosive sublimate, for instance, would be most inappropriate as a hardening agent.

32. The Practice of Hardening—Hints and Cautions.—Employ *in general* a *relatively large volume* of hardening liquid, and change it very frequently. If the volume of liquid be insufficient, its composition will soon become seriously altered by the diffusion into it of the soluble substances of the tissues; and the result may be a macerating instead of a hardening liquid. Further, as soon as, in consequence of this diffusion, the liquid has acquired a composition similar in respect of the proportions of colloids and crystalloids contained in it to that of the liquids of the tissues, osmotic equilibrium will become established, and diffusion will cease; that is to say, the hardening liquid will cease to penetrate. This means, of course, maceration of internal parts. On the other hand, it appears that a certain slight proportion of colloids in the hardening liquid is favourable to the desired reaction, as it gives a better consistency to the tissues by preventing them from becoming brittle. Hence the utility of employing *a certain proportion* of hardening agent.

Hardening had better be done in tall cylindrical vessels, the objects being suspended by a thread, or muslin bag, or otherwise, at the top of the liquid. This has the advantage of allowing diffusion to take place as freely as possible, whilst any precipitates that may form fall harmlessly to the bottom.

In general *begin* hardening with a *weak reagent,* increasing the strength gradually, as fast as the tissues acquire a consistence that enables them to support a more energetic action of the reagent.

Let the objects be removed from the hardening fluid as soon as they have acquired the desired consistency.

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CHAPTER IV.

FIXING AND HARDENING AGENTS—MINERAL ACIDS AND THEIR SALTS.

33. Osmic Acid.—The tetroxide of osmium (OsO_4) is the substance commonly known as osmic acid, though it does not possess acid properties. It is a substance that it is exceedingly difficult to keep in use for any length of time. It is extremely volatile, and in the form of an aqueous solution becomes partially reduced with great readiness in presence of the slightest contaminating particle of organic matter. It is generally believed that the aqueous solutions are reduced by light alone, but this is not the case: they may be exposed to the light with impunity if dust be absolutely denied access to them. (See § 357.)

Great stress is laid by authors on the fact that the vapour of osmium is very irritating to mucous tissues. It is said that the slightest exposure to it is sufficient to give rise to serious catarrh, irritation of the bronchial tubes, laryngeal catarrh, conjunctivitis, etc. I have never myself suffered in this way, but there is no doubt that many persons do, and such susceptible subjects should be very careful in handling osmium in any form.

34. How to keep the Solutions.—The solution of osmic acid in chromic acid solution is not, like the solution in pure water, easily reducible, but may be kept without any special precautions. I therefore keep the bulk of my osmium in the shape of a 2 per cent. solution of osmic acid in 1 per cent. aqueous chromic acid solution. This solution serves for fixation by osmium vapours, and for making up solution of Flemming, which is the form in which osmium is most generally employed. A small quantity of osmic acid may also be made up in 1 per cent. solution in distilled water, and kept carefully protected from dust for use in special cases. Those who have to do a great deal of fixing by means of the vapours may also keep a supply of the solid oxide for this purpose.

Grübler and Hollborn now send out osmic acid in tubes containing *one tenth* of a gramme.

Cori (*Zeit. wiss. Mik.*, vi, 1890, p. 442) finds that solutions in distilled water keep perfectly if there be added to them enough permanganate of potassium to give a very slight rosy tint to the liquid. From time to time, as the solution becomes colourless, further small quantities of the salt should be added, so as to keep up the rosy tint.

Busch finds that the addition of sodium iodate hinders reduction (see § 40).

Pintner finds that a slight addition of corrosive sublimate has the same effect, *e.g.* ten drops of 5 per cent. solution of sublimate added to 100 c.c. of 1 per cent. solution of osmic acid (Mayer, *Grundzüge*, p. 26).

35. Regeneration of Reduced Solutions.—Bristol (*Amer. Nat.*, xxvii, 1893, p. 175) says that reduced solutions may be regenerated by oxidising them by means of peroxide of hydrogen. The reaction is stated to be identical with that which takes place in the bleaching of osmium-blackened tissues by peroxide. It is admitted that the tetroxide of osmium, OsO_4, is reduced by contact with organic matter into the deutoxide, OsO_2. Then $OsO_2 + 2H_2O_2 = OsO_4 + 2H_2O$.

According to Bristol, for regenerating 100 c.c. of 1 per cent. solution of osmic acid (*erratim* 10 per cent. in *Journ. Roy. Mic. Soc.* 1893, p. 564), ten to twenty drops of fresh peroxide solution should be added.

Kolossow (*Zeit. wiss. Mik.*, ix, 1892, p. 40) says that half-reduced solutions, so long as they have not lost their characteristic odour, may be clarified by the addition of a little powdered potash-alum.

But this is evidently only a process of *clarification*, not of regeneration, the alum acting mechanically by carrying down the suspended matter, as isinglass does in the "fining" of beer. Mayer finds that addition of common salt will produce the same effect.

36. Fixation by the Vapours.—Osmic acid is frequently employed in the form of vapour, and its employment in this form is indicated in most of the cases in which it is possible to expose the tissues directly to the action of the vapour. The tissues are pinned out on a cork, which must fit well into a wide-mouthed bottle in which is contained a little solid osmic acid (or a small quantity of 1 per cent. solution will do). Very small objects, such as isolated cells, are simply placed on a slide, which is inverted over the mouth of the bottle. They remain there until they begin to turn brown (isolated cells will generally be found to be sufficiently fixed in thirty seconds: whilst in order to fix the deeper layers of relatively thick objects, such as retina, an exposure of several hours may be desirable). It is well to wash the objects with water before staining, but a very slight washing will suffice. For staining, methyl-green may be recommended for objects destined for study in an aqueous medium, and, for permanent preparations, alum-carmine, picrocarmine, or hæmatoxylin.

In researches on nuclei, it may be useful to employ the vapours of a freshly prepared mixture of osmic and formic or acetic acid (Gilson, *La Cellule*, i, 1885, p. 96).

An apparatus for conveniently employing the vapours is described by ANDREWS (*Zeit. wiss. Mik.*, xiv. 1898, p. 448).

The reasons for preferring the process of fixation by vapour of osmium, where practicable, are that osmic acid is more highly penetrating when employed in this shape than when employed in solution, and produces a more *equal* fixation, and that the arduous washing out required by the solutions is here done away with. In many cases delicate structures are better preserved, all possibility of deformation through osmosis being here eliminated.

37. Fixation by Solutions.—Osmic acid is now very seldom used *pure* in the shape of solutions, as it has been found to give better results when combined with other ingredients, as in the mixture of FLEMMING. When, however, it is employed in pure aqueous solutions it is used in strengths varying from $\frac{1}{20}$ per cent. to 1 per cent. I should say myself that, as a rule, not more than 0·1 per cent., and *never more than* 0·5 per cent., should be used.

On account of the feeble penetrating power of osmic acid, the objects to be fixed should be *as small as possible.*

A little acetic or formic acid (0·5 to 1 per cent.) may generally with advantage be added to the solutions just before using.

The solutions should be kept protected from the light during the immersion of tissues. (This precaution is not necessary if Flemming's or Hermann's solution be used.) If the immersion is to be a long one the tissues must be placed with the solution in well-closed vessels, as osmium is very volatile.

The objects may be deemed to be fixed as soon as they have become brown throughout.

38. After-Treatment.—The excess of osmic acid must be well washed out before proceeding to any further steps in preparation; water should be used for washing. Notwithstanding the greatest care in soaking, it frequently happens that some of the acid remains in the tissues, and causes them to over-blacken in time, and in any case hinders staining. To obviate this blackening it has been advised to wash them out in ammonia-carmine or picro-carmine (not very effectual), or to soak them for twenty-four hours in a solution of bichromate of potash (Müller's solution or Erlicki's will do), or in 0·5 per cent. solution of chromic acid, or in Merkel's solution. The treatment with bichromate solutions has the great advantage of highly facilitating staining with carmine or hæmatoxylin. Max Schultze recommended washing, and mounting permanently in acetate of potash; but I believe the virtues attributed to this method are illusory. Fol has recommended treatment with a weak solution of carbonate of ammonia. But the best plan of all is to properly *bleach* the preparations (see "**Bleaching**"). This may be done by means of *peroxide of hydrogen*, which regenerates the osmium to osmic acid. Overton (*Zeit. wiss. Mik.*, vii, 1890, p. 10) finds that bleaching is completed in a few minutes in a mixture of 1 part commercial peroxide of hydrogen with 10 to 25 parts 70 per cent. alcohol. (The commercial peroxide, slightly acidulated with HCl, will keep well in the dark; but the mixture with alcohol must be made fresh for use.) According to Bristol (*Amer. Natural*, xxvii, 1893, p. 176) the peroxide acts best in the sun. Binet (*Journ. de l'Anat. et de la Physiol.*, xxx, 1894, p. 449) has successfully used permanganate of potash. Mönckeberg and Bethe (*Arch. Mik. Anat.*, liv, 1899, p. 135) have succeeded in satisfactorily restoring the staining susceptibility of osmium material by means of sulphurous acid (obtained by adding hydrochloric acid to bisulphite of sodium, 2 to 4 drops of the acid added to 10 c.c. of a 2 per cent. solution of the salt). But perhaps the most convenient method is the chlorate of potash method of MAYER (§ 587).

FOL (*Lehrb.*, p. 174) recommends a weak aqueous solution of ferricyanide of potassium. MAYER (*Grundzüge*, p. 29) objects that the ferricyanide only acts in aqueous solution, not in alcoholic. He has had good, though slow, results with peroxide of magnesium (from which the oxygen is set at liberty by means of an acid).

I find the sulphate of iron solution used in Benda's hæmatoxylin stain has a marked bleaching effect, and so also, though in a less degree, the iron alum of Heidenhain's process.

The same stains recommended for objects fixed by the vapours will be found useful here. For sections, of course, in both cases safranin and other anilin stains may be employed with advantage, as may hæmatoxylin.

39. Characters of the Fixation with Osmic Acid.—In general osmic acid, especially when used in the form of vapour, fixes protoplasm faithfully, nuclei badly. It is pre-eminently a fixative of the *hyaloplasm* or enchylema of cells (see §§ 28 and 654). The *penetrating power* of the solutions is *very low*, so that if any but very small pieces of tissue be taken the outer layers become over-fixed before the action of the reagent has penetrated to the deeper layers. Over-fixed cells have a certain homogeneous, glassy, or colloid look, and are unfit for study (see § 28). For this reason it is important to avoid using stronger solutions than is necessary. Care should be taken not to draw conclusions as to the structure of cells from over-fixed specimens, and attention should be confined to cells four or five layers deeper down, which will generally be found to present the required intensity of fixation.

Osmic acid stains certain fatty bodies black; it should therefore be avoided for tissues in which much fat is present; or, if not, the preparations should be subsequently very thoroughly bleached, or the blackened fat may be afterwards dissolved out by means of oil of turpentine (§ 707).

According to ALTMANN, STARKE, and HANDWERCK, only free oleic acid and olein are directly blackened by osmic acid; stearin and palmitin, and stearic and palmitic acid are only browned by it, with an after-blackening which is produced by subsequent treatment with alcohol. Neither reaction occurs with the fatty bodies in the solid state, and can only be obtained when they are either in a state of fusion or solution (from the paper of HANDWERCK in *Zeit. wiss. Mik.*, xv, 1898, p. 177). See also LOISEL, C. R., *Soc. Biol.*, 1903, p. 826.

40. Osmic Mixtures.—The chief osmic mixtures are those of FLEMMING and of HERMANN, for which see §§ 46, 47, and 50. The following, however, may be mentioned here.

RANVIER ET VIGNAL (RANVIER, *Leç. d'Anat. Gén.*, p. 76; VIGNAL, *Arch. de Physiol.*, 1884, p. 181) take equal volumes of 1 per cent. osmic acid and 90 per cent. alcohol (freshly mixed). They wash out in 80 per cent. alcohol, then wash with water and stain for forty-eight hours in picro-carmine or hæmatoxylin. Viallanes has applied this method to the histology of insects.

KOLOSSOW (*Zeit. wiss. Mik.*, v, 1888, p. 51) has recommended a 0·5 per cent. solution of osmic acid in 2 or 3 per cent. solution of nitrate or acetate of uranium, as having a greatly enhanced penetrating power.

He has more lately (op. cit., ix, 1892, p. 39) recommended for the same reason a mixture of 50 c.c. absolute alcohol, 50 c.c. distilled water, 2 c.c. concentrated nitric acid, and 1 to 2 grm. osmic acid. This mixture is said to keep indefinitely in a cool place.

NICOLAS (*Intern. Monatsschr.*, 1891, p. 3) adds ½ per cent. of osmic acid to nitric acid of 3 per cent. I have employed a similar mixture and not had good results, though I find the mixture keeps perfectly.

BUSCH (*Neurol. Centralb.*, xvii, 1898, No. 10, p. 476; *Zeit. wiss. Mik.*, xv, p. 373) holds that the penetration of osmic acid is enhanced by combining it with iodate of sodium, which by hindering its too rapid decomposition in the tissues ensures a more energetic action in the deeper layers. He adds 3 per cent. of sodium iodate to a 1 per cent. solution of osmic acid.

This mixture appears to me rational, whilst the above-mentioned mixtures with alcohol do not. Alcohol is a reducing agent, and therefore incompatible with so easily reducible a substance as osmic acid, which should rather be combined with oxidising agents. Now, sodium iodate is a powerful oxidising agent, and so far seems quite indicated.

41. Chromic Acid.—Chromic anhydride, CrO_3, is found in commerce in the form of red crystals that dissolve readily in water, forming chromic acid, H_2CrO_4. These crystals are very deliquescent, and it is therefore well to keep the acid in stock in the shape of a 1 per cent. solution. Care must be taken not to allow the crystals to be contaminated by organic matter, in the presence of which the anhydride is readily reduced into sesquioxide.

Chromic acid is generally employed in aqueous solution. Some observers (KLEIN; URBAN PRITCHARD; PERÉNYI) have recommended alcoholic solutions; but this would appear to be an irrational practice. For in the presence of alcohol chromic acid has a great tendency to become reduced to chromous oxide or sesquioxide, neither of which appears to have any fixing power.

The most useful strengths in which it is employed in aqueous solution are from 0·1 to 1·0 per cent. for a period of immersion of a few hours (structure of cells and ova). For nerve tissues weaker solutions are taken, $\frac{1}{50}$ to $\frac{1}{8}$ per cent. for a few hours. Stronger solutions, such as 5 per cent., should only be allowed to act for a few seconds.

Washing out.—The general practice is to wash out very thoroughly with water (by preference running water, for many hours), before bringing into alcohol or any staining liquid. The reason for this practice is that when objects that have been treated by chromic acid or a chromate are put direct into alcohol it is found that after a short time a fine precipitate is thrown down on the surface of the preparations, thus forming a certain obstacle to the further penetration of the alcohol. Previous washing by water does not prevent the formation of this precipitate, and changing the alcohol does not prevent it from forming again and again. It has, however, been found by HANS VIRCHOW (*Arch. mik. Anat.*, xxiv, 1885, p. 117) that the formation of this precipitate may be entirely prevented by simply keeping the preparations *in the dark*. The alcohol becomes yellow as usual (and should be changed as often as this takes place), but no precipitate is formed. If this precaution be taken, previous washing with water may be omitted, or at all events greatly abridged.

MAYER (*Grundzüge*, p. 32) proceeds as follows:—The fixed material is merely rinsed in water and brought direct into 70 per cent. alcohol. It is washed therein, preferably in the dark, until after several changes the alcohol remains colourless. It is then either passed through higher alcohols and embedded in paraffin, the chromous oxide (or whatever chrome compound it may be that is present in the tissues) being removed from *the sections* after these are made; or this necessary removal is performed at once. If this be preferred, the material is brought into sulphuric acid diluted with twenty volumes of water, or into nitric acid diluted with ten volumes of water. After at most a few hours therein, it will have become of a light greyish green, and on removal of the acid may be readily stained. If it be preferred to treat *the sections*, it is sufficient to put them into the usual hydrochloric acid alcohol (four to six drops of HCl to 100 c.c. of 70 per cent. alcohol), in which after a short time they become almost white, and will stain excellently with any of the usual stains. So also EDINGER (*Zeit. wiss. Mik.*, i, 1884, p. 126; nitric acid 1 : 20 for five minutes). UNNA (*Arch. mik. Anat.*, xxx, 1887, p. 47) holds that the chrome is present in the tissues in the form of chromic chromate, and removes it by treatment with peroxide of hydrogen. OVERTON (*Zeit. wiss. Mik.*, vii, 1890, p. 9) employs a weak solution of sulphurous acid, which converts it into a sulphate. See also the directions for bleaching osmic acid preparations, § 38.

Tissues that have been fixed in chromic acid may be stained in aqueous solutions, as water does not have an injurious effect on them.

The best stain for chromic material that has not been treated by Mayer's special process, or by a similar one, is hæmatoxylin, or, for sections, the basic tar colours.

Chromic acid is not a very penetrating reagent, and for this reason, as well as for others, is now seldom used pure for fixing, but plays an important part in the mixtures described below, of which the chief is certainly the mixture of Flemming.

For prolonged hardening it is generally employed in strengths of $\frac{1}{5}$ per cent. to $\frac{1}{2}$ per cent., the immersion lasting a few days or a few weeks, according to the size and nature of the object. Mucous membrane, for instance, will harden satisfactorily in a few days; brain will require some six weeks.

Large quantities of the solution must be taken (at least 200 grammes for a piece of tissue of 1 centimetre cube—Ranvier).

In order to obtain the best results you should not employ portions of tissue of more than an inch cube. For a human spinal cord you should take two litres of solution, and change it for fresh after a few days. Six weeks or two months are necessary to complete the hardening.

I think it is frequently useful to add a little glycerin to the hardening solution ; there is less brittleness.

The solution should be taken weak at first, and the strength increased after a time. The objects should be removed from the solution as soon as they have acquired the desired consistency, as if left too long they will become brittle. They may be preserved till wanted in alcohol (95 per cent.). It is well to wash them out in water for twenty-four or forty-eight hours before putting them into the alcohol. After a time they generally become green in the alcohol. They may be *bleached* if desired.

Chromic acid is a most powerful and rapid hardening agent. (By it you may obtain in a few days a degree of hardening that you would hardly obtain in as many weeks with bichromate, for instance.) It has the defect of a great tendency to cause brittleness.

42. Chromic Acid and Spirit (URBAN PRITCHARD, *Quart. Journ. Mic. Sci.*, 1873, p. 427).—Chromic acid, 1 part; water, 20 parts; rectified spirit, 180 parts. The colour of the solution soon becomes brown. If after a few days it turns semi-gelatinous, it should be changed for fresh. From a week to ten days is required to harden such tissues as retina, cochlea, etc., for which this fluid used to be considered particularly well adapted.

A mixture of 2 parts of $\frac{1}{6}$ per cent. chromic acid solution with 1 part of methylated spirit was much used by KLEIN (*Quart. Journ. Mic. Sci.*, 1878, p. 315) in his investigations into the structure of cells and nuclei, and found to give better results than other reagents.

Both these mixtures are seemingly irrational (see § 41). MAYER (*Grundzüge*) remarks on Pritchard's formula: "An altogether silly receipt; after a short time the mixture has lost its acid reaction, and then only the alcohol can act." See also the remarks on the mixture of PERÉNYI, § 52.

A still more irrational mixture with oxalic acid has been propounded by GRAF (*Cont. Path. Inst. New York State Hosp.*, 1898, No. 15; see *Grundzüge*, 2nd ed., p. 34).

43. Chromo-acetic Acid (FLEMMING, *Zellsbz., Kern u. Zel lth.* p. 382).

Chromic acid . 0·2 to 0·25 per cent.
Acetic acid . . 0·1 per cent. in water.

Flemming found this the best reagent for the study of the *achromatic* elements of karyokinesis. You can stain with hæmatoxylin, or the basic anilin dyes.

The following has been recommended as a good fixing and hardening mixture for Annelids in general, and probably for other forms, by EHLERS (I do not know whether it has been published elsewhere) :—To 100 c.c. of chromic acid of 0·5 to 1 per cent. add from 1 to 5 drops of glacial acetic acid. The proportion of acetic acid indicated is said to be sufficient to counteract any tendency to shrinkage due to the chromic acid.

Similar to this is the "chromo-acetic acid, No. 1," of LO BIANCO (*Mitth. Zool. Stat. Neapel*, ix, 1890, p. 443), viz. 1 part 50 per cent. acetic acid and 20 parts 1 per cent. chromic acid, which is found very useful for fixing marine animals.

44. Chromo-formic Acid (RABL, *Morph. Jahrb.*, x, 1884, pp. 215, 216).—Four or five drops of concentrated formic acid are added to 200 c.c. of 0·33 per cent. chromic acid solution. The mixture must be freshly prepared at the instant of using. Fix for twelve to twenty-four hours, wash out with water. Used by Rabl for the study of karyokinesis.

45. Chromo-osmic Acid (MAX FLESCH, *Arch. mik. Anat.*, xvi, 1879, p. 300).—This mixture (osmic acid, 0·10; chromic acid, 0·25; water, 100·0) may for almost all purposes be considered to be superseded by that of Flemming, § 46.

LO BIANCO (*Mitth. Zool. Stat. Neapel.*, ix, 1890, p. 443) employs for marine animals a mixture of 1 part 1 per cent. osmic acid and 50 parts 1 per cent. chromic acid.

46. Chromo-aceto-osmic Acid (FLEMMING, *Zellsubstanz, Kern und Zelltheilung*, 1882, p. 381), FIRST or WEAK formula:

Chromic acid . .	0·25 per cent.	in water.
Osmic acid . . .	0·1 „	
Glacial acetic acid . .	0·1 „	

This mixture is now almost entirely superseded by the strong mixture, next §. If a weak mixture be required, one having approximately the same composition as the weak liquid may easily be made by diluting the strong liquid with 2 vols. of water—the proportion of osmic and chromic acid will then be only slightly changed, that of the acetic acid not injuriously increased.

FOL (*Lehrb. d. vergl. mik. Anat.*, 1884, p. 100) recommends the following variant:

1 per cent. chromic acid . . .	25 vols.
1 per cent. osmic acid	2 „
2 per cent. acetic acid	5 „
Water	68 „

—that is to say, a mixture much weaker in osmium than Flemming's.

A mixture still weaker than this in osmium, viz. with 1 vol. osmic acid solution, instead of 2, has been recommended by Cori (*Zeit. wiss. Mik.*, vi, 1890, p. 441).

47. Chromo-aceto-osmic Acid (Flemming, *Zeit. wiss. Mik.*, 1, 1884, p. 349), SECOND OR STRONG formula:

1 per cent. chromic acid	15 parts.
2 per cent. osmic acid	4 ,,
Glacial acetic acid	1 part.

If 2 per cent. osmic acid solution should not be at hand, you may conveniently make the mixture by taking—

10 per cent. chromic acid	15 parts.
1 per cent. osmic acid	80 ,,
Glacial acetic acid	10 ,,
Water	95 ,,

If this mixture be kept in stock in large quantities, it may go bad, probably on account of the large proportion of organic acid contained in it. I therefore recommend that it be made up from time to time from stock solutions, in which the osmic acid is kept separate from the acetic acid. The proportions being as follows:—

CrO_3	0·15
OsO_4	0·08
Acid. acet.	1·00
Aq.	19·00

you may make up and keep separately—

(A)	1 per cent. chromic acid	11 parts,
	Distilled water	4 ,,
	Glacial acetic acid	1 part,

and (B) a 2 per cent. solution of osmic acid in 1 per cent. chromic acid solution, and when required, mix four parts of A with one of B; or, if you prefer it, you may keep the osmic and chromic acid ready mixed in the proportions given, and add 5 per cent. of acetic acid at the moment of using.

Merk (*Denksch. Math. Naturw. Cl. K. Acad. Wiss. Wien*, 1887; cf. *Zeit. f. wiss. Mik.*, v. 1888, p. 237) proposes to make up separately (A)

2 per cent. chromic	7·5 parts,
Water	3·5 ,,
Acetic acid	1 part,

and (B), some 1 per cent. osmic acid solution, and to mix for use 12 parts of A with 8 of B. But this plan leaves you in the old difficulty of keeping your osmium in aqueous solution.

According to Flemming, osmium being very volatile it will be found that solutions that have been long in use no longer contain the proper proportion of that ingredient, and the hardening action being thus weakened, the swelling action of the acetic acid may be insufficiently controlled.

More recently, Flemming has been making up the mixture with only 2 parts of the osmic acid instead of 4, and has spoken of this modification as "weaker osmium mixture" (Meves, in *Encyclop. d. Mik. Technik*, pp. 388, 389).

Podwyssozki recommends (for glands especially) the following modification:

1 per cent. CrO_3 dissolved in 0·5 per cent. solution of corrosive sublimate	15 c.c.
2 per cent. osmic acid solution	4 c.c.
Glacial acetic acid	6 to 8 drops.

The sublimate is said to augment the penetration of the osmium, but is unfavourable to staining (Ziegler's *Beiträge z. path. Anat.*, i, 1886; *Zeit. wiss. Mik.*, iii, 1886, p. 405).

Flemming's liquid may, without inconvenience, be allowed to act for many hours or days, or according to some workers even weeks or months; but this exaggerated fixation is clearly only justifiable in very special cases, if at all. Wash out very thoroughly in water (running, 24 hours, or treat as directed for chromic acid § 41). Stain with alum hæmatoxylin if you wish to stain *in toto* (staining in this way with other reagents is possible, but difficult). Stain sections with safranin or other basic coal-tar colour, or with hæmatoxylin.

For fixing with the *strong* mixture you need only take a bulk of liquid of some 4 times the volume of the objects (but with the weak mixture the proportion should be increased). This mixture is a first-rate fixative of cellular structures, both as regards their *preservation* and as regards their *optical differentiation* (§ 28). But it must be properly used, and not applied to objects for which it is not fitted. For instance, its *power of penetration* is *extremely bad;* it will not fix properly, even in a loose-celled tissue, through more than a layer of about five cells thick. That is to say, it has the defect of easily causing over-fixation of superficial cells, and, owing to its defective penetration, insufficient fixation of deep-lying ones. It is therefore suitable only for *very small* objects or for *very small* pieces of tissue, such as suffice for cytological or histological work. It is not suitable at all for voluminous objects, such as the organological anatomist and the embryologist so frequently have to do with. It has not the character of a *general* reagent. As a matter of fact it was recommended by FLEMMING in the first instance merely for a very special purpose, the hunting for karyokinetic figures, and not for general purposes.

It may be used for prolonged hardening, *e. g.* of nervous tissue, and is a very good reagent for the purpose.

Fat is blackened (or browned) by it (see §§ 39 and 707). Chromatin is mordanted by it for basic anilin dyes, enabling them to give peculiarly sharp and powerful stains (see §§ 292, 295, 298 *et seq.*).

48. Osmic Acid and Bichromate.—ALTMANN (*Die Elementarorganismen*, Leipzig, 1890; *Zeit. wiss. Mik.*, vii, 1890, p. 199) recommends a mixture of equal parts of 5 per cent. solution of bichromate of potash and 2 per cent. solution of osmic acid for the demonstration of his granula. The bichromate, he says, ought not to contain any free chromic acid, and the mixture is best prepared freshly when required.

LO BIANCO (*Mitth. Zool. Stat. Neapel*, ix, 1890, p. 443) employs for marine animals a mixture of 100 c.c. of 5 per cent. solution of bichromate and 2 c.c. of 1 per cent. osmic acid.

Evidently, I think, the addition of acetic acid to either of these mixtures is in most cases indicated. This step has, in fact, been taken by HOEHL (*Arch. Anat. Phys., Anat. Abth.*, 1896, p. 31), who recommends a mixture of 80 c.c. of 3 per cent. bichromate, 20 c.c. of 1 per cent. osmic acid, and 2 c.c. of glacial acetic acid.

49. Osmic, Bichromate, and Platinic Mixture (LINDSAY JOHNSON'S Mixture).—Latest formula, 1895, communicated by Dr. Lindsay Johnson:

Bichromate of potash (2·5 per cent.) .	70 parts.
Osmic acid (2 per cent.)	10 „
Platinum chloride (see § 81) (1 per cent.)	15 „
Acetic or formic acid.	5 „

HENNEGUY, who has worked a great deal with this reagent, and recommends it highly, says (*Leçons sur la Cellule*, Paris, Carré, 1896, p. 61) that it is well only to add the acetic or formic acid just before using, as it frequently provokes a spontaneous reduction of the osmium and platinum to such an extent that the mixture becomes quite black.

This mixture was invented for the preliminary hardening of retina, being allowed to act for two hours only, and then being followed by final hardening in pure bichromate solution. But it has proved applicable to other structures, and gives excellent results. The function of the osmic acid in the present formula is to enhance the hardening energy of the mixture. Dr. Lindsay Johnson writes me that "it greatly reduces the length of time necessary for hardening, three days being the time from removal of the organ to its being in celloidin under dilute spirit."

This mixture may be used for fixing, in some cases with the best results. HENNEGUY (*l. c.*) says it contracts the more spongy sorts of protoplasm less than mixture of FLEMMING. I think highly of it—for certain objects.

50. Platino-aceto-osmic Acid (HERMANN'S **solution**).—HERMANN (*Arch. mik. Anat.*, xxxiv, 1889, p. 58) substitutes 1 per cent. platinum chloride (see § 81) for the chromic acid in Flemming's *strong* formula for chromo-aceto-osmic acid (§ 47), the other ingredients either remaining as before, or the osmium being diminished one half; thus, 1 per cent. platinum chloride 15 parts, glacial acetic acid one part, and 2 per cent. osmic acid either 4 parts or only 2 parts. Hermann found that protoplasmic structures are thus better preserved than with the chromic mixture.

The after-treatment and staining should be the same as for objects treated with Flemming's solution.

The action of this fixative is, roughly, similar to that of Flemming's. Like Flemming's, it mordants chromatin for staining with "basic" colours, with which it affords equally fine nuclear stains. But, owing to the platinum in it, it diminishes more than Flemming's the colorability of tissues with "acid" colours, so that it is *extremely difficult* to obtain *good plasma stains* after its action. It causes a notable shrinkage in chromatin. It gives a *full* fixation (§ 654) of cytoplasm, to which it gives a much more fine-grained aspect than liquid of Flemming does, and this effect may be so pronounced as to amount to a defect, minute detail being masked by the general homogeneity of the image.

51. **Nitric Acid** (ALTMANN, *Arch. Anat. Phys.*, 1881, p. 219).—Altmann employs for fixing dilute nitric acid, containing from 3 to 3½ per cent. pure acid. Such a solution has a sp. gr. of about 1·02. Stronger solutions have been used, but do not give such good final results. After extensive trial I find ALTMANN'S solution to be a second-rate reagent, giving a weak and thin fixation.

HIS (*ibid.*, 1877, p. 115) recommended a 10 per cent. solution. Flemming at one time employed solutions of 40 to 50 per cent. for the ova of Invertebrates. This, of course, has the advantage of a very rapid fixing action.

TELLYESNICZKY (*Arch. mik. Anat.*, lii, 2, 1898, p. 222) thinks that "for general cell-fixing" the proper strength is 2 per cent. to 2½ per cent., as stronger grades act too energetically on the superficial layers. His results, or the interpretation of them, are therefore not concordant with mine.

Nitric acid has the valuable property of *hardening yolk without making it brittle.*

Pure water should in no case be used for washing out after nitric acid; the preparations should be brought direct into alcohol, as recommended by Altmann. Some persons take absolute alcohol, but I should say 70 per cent. is more generally indicated. Rabl has employed for washing out a 1 or 2 per cent. solution of alum.

For *prolonged hardening*, strengths of from 3 to 10 per cent. are sometimes employed. A strength of 12 per cent., allowed to act for two or three weeks, is said to afford very tough preparations of the encephalon.

BENDA (*Verh. Anat. Ges.*, 1888; *Ergeb. d. Anat.*, i, 1891, p. 7) fixes for twenty-four to forty-eight hours in 10 per cent. nitric acid, and then brings the preparations direct into a cold saturated solution of bichromate of potash diluted with three vols. of water. After a few hours this solution is changed for a stronger one, and the strength is gradually increased in such a manner as to arrive at a concentration of one vol. of the saturated solution to one of water in two or three days (or, for encephalon and spinal cord only in fourteen days). This process, which is of general applicability (except in so far as epidermis, especially that of embryos, may be loosened by the nitric acid), is said to furnish very tough preparations.

Fol's Mixture.—Three vols. of nitric acid, with 97 vols. of 70 per cent. alcohol (verbally communicated to me by Prof. Fol).

52. **Chromo-nitric Acid** (PERÉNYI'S formula, *Zool. Anzeig.*, v, 1882, p. 459):

4 parts 10 per cent. nitric acid.
3 parts alcohol.
3 parts 0·5 per cent. chromic acid.

These are mixed, and after a short time give a fine violet-coloured solution.

The objects are immersed for four to five hours, and then passed through 70 per cent. alcohol (twenty-four hours), strong alcohol (some days), absolute alcohol (four to five days). They are then fit for cutting. The advantage of the process is said to be, amongst others, that segmentation spheres and nuclei are perfectly fixed, the ova do not become porous, and cut like cartilage.

For a special formula for embryological purposes see the paper quoted.

This liquid has been for a long time in great vogue not only for embryological purposes, but for general work and cytological work. But opinions are divided as to its merits. I myself have extensively used it for preparing specimens for dissection and for museum specimens, and have found it admirable for these purposes. But specimens made to test its value from a cytological point of view have given me only second-rate results.

Mayer contributes the following note on this subject to the *Grundzüge*, 1st ed. (p. 34) :—"Perényi's mixture does not appear to have been hitherto considered from a chemical point of view. It is, however, easy to see that as soon as the mixture has become violet, the chromic acid no longer exists in it as such, but has been changed into chromic oxide. At the expense of this oxide the alcohol becomes oxidised, and in consequence of the presence of the nitric acid becomes partially converted into nitric ether. Consequently the liquid is reduced essentially to a mixture of alcohol of at most 30 per cent. strength with about 5 per cent. of nitric acid. An analogous mixture made by omitting the chromic acid preserves, according to my experiments, in just the same way as Perényi's,—that is, just as so weak an acid alcohol can be expected to preserve, and that is rather ill than well. Objects, it is true, do not shrink in it ; indeed, they rather swell, sometimes to a marked degree. And in fact, observers are not wanting who entirely reject it for the fixation of *ova*. See the strange results of Cholodkovsky in the embryology of *Blatta*, on which I have commented in *Zool. Jahresbericht*, 1891 (Arthropoda, p. 61), and which Wheeler and Heymons have later (*ibid.*, 1893, p. 71, and 1895, p. 61) expressly referred to the fixing liquid. In any case the end is more simply attained by taking simply *acid alcohol*, as recommended by me so long ago as 1880 (*Mitth. Z. Stat. Neapel*, ii, p. 7), which may be taken weaker or stronger according to the nature of the objects."

I fancy the reaction is much more complicated than sketched out by Mayer. Possibly aldehyde is formed, and helps in the hardening. At all events, numerous workers speak highly of the mixture, for certain purposes.

53. Chromic Acid and Platinum Chloride (MERKEL'S *Macula lutea des Menschen*, Leipzig, 1870, p. 19).—Equal volumes of 1·400 solution of chromic acid and 1·400 solution of platinum chloride (see § 81). Objects should remain in it for several hours or even days, as it does not harden very rapidly. After washing out with alcohol of 50 per cent. to 70 per cent., objects stain excellently, notwithstanding the admixture of chromic acid. If objects that have been fixed by osmic acid be put into it for some hours, blackening is said to be effectually prevented.

This is an excellent hardening medium for delicate objects. Merkel states that he allowed from three to four days for the action of the fluid for the retina; for Annelids Eisig employs an immersion of three to five hours, and transfers to 70 per cent. alcohol; for small leeches Whitman finds one hour sufficient, and transfers to 50 per cent. alcohol.

A similar mixture, with the addition of 0·25 to 0·1 per cent. of acetic acid, is recommended by BRASS for Protozoa; and LAVDOWSKY has used for nuclei a mixture of 10 parts of 1 per cent. chromic acid, 5 of 1 per cent. platinum chloride, and 100 of 5 per cent. acetic acid.

Whitman recommends, for the hardening of pelagic fish ova, a stronger mixture (due, I believe, to Eisig), viz.—

0·25 per cent. solution of platinum chloride .	1 vol.
1 per cent. solution of chromic acid . .	1 ,,

The ova to remain in it one or two days (WHITMAN, *Methods in Micro. Anat.*, p. 153).

Salts.

54. Chromates.—The chromates are amongst the oldest and best tried of hardening agents. The bichromate of potash especially was at one time universally employed for hardening all sorts of tissues, and a great amount of classical work has been done with it.

About eighteen years ago, however, bichromate fell into disrepute in consequence of a criticism on its action made by FLEMMING. FLEMMING pointed out (*Arch. mik. Anat.*, xviii, 1880, p. 352) that though it preserves cytoplasm well it causes chromatin to swell, and therefore should not be employed *for the study of nuclei.* His readers, *plus royalistes que le roi,* took that to be a reason for abandoning it altogether, and from that time until quite recently it has lain in the cold shadow of neglect for almost all purposes except the hardening of nervous tissue. Erroneously, for, *duly corrected with acetic acid,* it affords a correct and fine fixation of nuclei; whilst preserving *hyaloplasm and its inclusions,* secretions, etc., much better than chromic acid (sometimes overmuch) see § 654.

For an elaborate study of the action of chrome salts on nucleus and cytoplasm, see BURCKHARDT, *La Cellule,* xii, 1897, p. 335. He finds that the bichromates of sodium, ammonium, magnesium, strontium, and zinc have the same destructive action on nuclei that the bichromate of potassium has; but that the bichromates of barium, calcium, and copper have not. The practical results of his researches may be summed up as follows: *Acetic acid ought always to be added,* not only to ensure the correct fixation of nuclei, but also to enhance penetration and the good preservation of cytoplasm.

The following is recommended by him as a good combination for the fixation both of cytoplasm and nucleus:

Bichromate of barium, 4 per cent. solution .	60 vols.
Bichromate of potassium, 5 per cent. solution	30 ,,
Glacial acetic acid	5 ,,

(Instead of the barium you may take 4 per cent. solution of bichromate of calcium, or 6 per cent. solution of bichromate of copper.)

For the demonstration of the achromatic figure of cell division he recommends—

Chromic acid, 1 per cent. solution . .	60 vols.
Bichromate of potassium, 5 per cent. solution .	30 ,,
Glacial acetic acid	5 ,,

55. Bichromate of Potash.—Perhaps the most important of all known *hardening* agents, *sensu stricto.* It hardens slowly, much more so than chromic acid, but it gives an incomparably better consistency to the tissues, and it has not the same tendency to make them brittle if the reaction be prolonged. They may remain almost indefinitely exposed to its action without much hurt.

The strength of the solutions employed is from 2 to 5 per cent. As with chromic acid, it is extremely important to begin with weak solutions and proceed gradually to stronger ones. About three weeks will be necessary for hardening a sheep's eye in solutions gradually raised from 2 to 4 per cent. Spinal cord requires from three to six weeks; a brain at least as many months.

After hardening, the objects should be well soaked out in water before being put into alcohol, or be treated as directed for chromic acid, § 41. They had better be kept in the dark when in alcohol (*see* § 41). (Böhm and Oppel [*Taschenbuch*, 3 Auf., 1896, p. 22] fix in the dark). *If you wish to have a good stain with carmine you should not put the objects into alcohol at all, even for a second, until they have been stained.*

You may stain either with carmine or hæmatoxylin, as well as with tar colours.

Bichromate objects have an ugly yellow colour which cannot be removed by mere soaking in water. It is said that it can be removed by washing for a few minutes in a 1 per cent. solution of chloral hydrate. Gierke, however, says that this treatment is prejudicial to the preservation of the tissues.

Prof. Gilson writes me that alcoholic solution of sulphurous anhydride (SO_2) is very convenient for the rapid decoloration of bichromate objects. A few drops suffice. *See* also § 41, and "Bleaching."

To facilitate staining with hæmatoxylin, Wolff (*Zeit. wiss. Mik.*, xv, 3, 1899, p. 311) first stains in Boehmer's hæmatoxylin for twenty-four hours, and then for a few minutes in the same hæmatoxylin to which has been added 1 drop per watch-glassful of 5 per cent. solution of oxalic acid.

The simple aqueous solution of bichromate is hardly to be recommended as a *fixing* agent, because not only does it not preserve nuclei, but also because it penetrates very slowly. The first of these defects may be overcome entirely, the second to some extent by addition of acetic acid; whence the liquid of Tellyesniczky, next §.

56. Acetic Bichromate (Tellyesniczky, *Arch. mik. Anat.*, lii, 1899, p. 242).—Tellyesniczky finds that the addition of acetic acid to bichromate not only suffices to ensure correct fixation of nuclei (see § 54), but also is favourable as regards its action on cytoplasm. He recommends the following formula, it being understood that the proportions may be varied if desired :

Bichromate	3 grms.
Glacial acetic acid	5 c.c.
Water	100 ,,

Smaller objects to remain in the fluid for one or two days, larger ones longer. Wash well in plenty of water, and pass through alcohols of increasing strength, beginning with 15 per cent. The results may be compared with those of liquid of Zenker, with the advantage that the ulterior treatment is greatly simplified.

Mixtures of bichromate with osmic acid have been given above, §§ 48 and 49.

57. Müller's Solution.—

Bichromate of potash	2–2½ parts.
Sulphate of soda	1 part.
Water	100 parts.

The duration of the reaction is about the same as with the simple solution of chromic salt.

This fluid was very highly in vogue for many years, but seems lately to be much less used. Recent authors find its action to be identical with that of plain bichromate, and doubt whether the sulphate in it has any effect whatever as regards its hardening properties. I fancy, however, that the superiority of this mixture over the simple bichromate solution is not illusory, and is due to the formation in it of a trace of free chromic acid. Fol says that for mammalian embryos, for which it has been recommended, it is worthless.

58. Erlicki's Solution (*Warschauer med. Zeit.*, xxii, Nos. 15 and 18 ; *Progrès Médical*, 1897, No. 39).—

Bichromate of potash	2·5 parts.
Sulphate of copper	1·0 part.
Water	100·0 parts.

Here the addition of the cupric sulphate is intelligible. This salt is itself a hardening agent of some energy, and may well serve to reinforce the somewhat slow action of the bichromate. As a matter of fact, "Erlicki" hardens very much more rapidly than either simple bichromate or Müller's solution. A spinal cord may be hardened in it in four days at the temperature of an incubator, and in ten days at the normal temperature (Fol, *Lehrb. d. vergl. mik. Anat.*, p. 106). Human embryos of several months may be conveniently hardened in it.

Nerve-centres that have been hardened in Erlicki's fluid frequently contain dark spots with irregular prolongations, simulating ganglion-cells. These were at one time taken to be pathological formations, but they are now known to consist of precipitates formed by the action of the hardening fluid. They may be removed by washing with hot water, or with water slightly acidified with hydrochloric acid, or by treating the specimens with 0·5 per cent chromic acid before putting them into alcohol (Tschisch, *Virchow's Arch.*, Bd. xcvii, p. 173: Edinger, *Zeit. wiss. Mik.*, ii, p. 245; Loewenthal, *Rev. méd. de la Suisse romande*, 6me année, i, p. 20).

58a. Dekhuyzen's Liquids (*C. R. Acad. Sci.*, cxxxvii, 1903, pp. 415 and 445).—(a) 250 c.c. of 2·5 per cent. sol. of bichromate in sea-water, 25 c.c. of 6·3 per cent. nitric acid, and 54 c.c. of 2 per cent. osmic acid. For general use with marine animals.

(b) 173·1 c.c. of the bichromate sol. and 26·9 of 2 per cent. sol. of osmic acid. For objects containing calcareous elements that it is desired to preserve.

These liquids are stated to be isotonic with sea-water.

59. Bichromate and Cupric Sulphate (Kultschitzky, *Zeit wiss. Mik.*, iv, 1887, p. 348).—A saturated solution of bichromate of potash and sulphate of copper in 50 per cent. alcohol, to which is added at the instant of using a little acetic acid, five or six drops per 100 c.c.

To make the solution, add the finely powdered salts to the alcohol in excess, and leave them together *in total darkness*, for twenty-four hours.

Fix for twelve to twenty-four hours *in the dark*, otherwise the salts will be precipitated. Then treat with strong alcohol for twelve to twenty-four hours, and make sections.

60. Bichromate and Sublimate (Kultschizky, *Arch. f. mik. Anat.*, xlix, 1897, p. 8).—Two parts bichromate, ¼ part corrosive sublimate, 50 parts 2 per cent. acetic acid, and 50 parts 96 per cent. alcohol. As part of the bichromate precipitates, the mixture should be filtered after twenty-four hours. Tissues of vertebrates may remain in it for four to six days.

61. Bichromate and Sublimate (Lavdowsky, *Zeit. wiss. Mik.*, xvii, 1900, p. 301).—500 c.c. of 1 per cent. acetic acid, 20 to 25 g. bichromate, and 5 to 10 c.c. saturated solution of sublimate in water. Fix for two or three days, wash with water, and harden for two to seven days in alcohol of 90 to 95 per cent. See also § 78.

62. Bichromate of Ammonia.—This salt is in considerable favour for hardening. Its action is very similar to that of the potassium salt Fol says that it penetrates somewhat more rapidly, and hardens somewhat more slowly. It should be employed in somewhat stronger solutions, up to 5 per cent.

63. Neutral Chromate of Ammonia is preferred by some anatomists. It is used in the same strength as the bichromate. Klein has recommended it for intestine, which it hardens, in 5 per cent. solution, in twenty-four hours.

64. Bichromates and Alcohol.—Mixtures of either bichromate of potash or of ammonia with alcohol may be employed, and have a more rapid action than the aqueous solution. Thus HAMILTON takes for hardening brain a mixture of 1 part methylated spirits with three parts of solution of Müller (see the chapter on the "Central Nervous System" in Part II ; see also KULTSCHITZKY's Mixture, *ante*, § 59). Preparations should be kept in the dark during the process of hardening in these mixtures.

65. Bichromates (or Monochromates) and Formol.—See § 120 and "*Neurological Methods.*"

66. Cupric Sulphate.—Not of general utility. See §§ 58, 59, and "*Siphonophora.*"

67. Alum.—Alum has been used for fixing purposes. After an extended experience of it, I only quote it in order to recommend that it be avoided at all costs.

68. Sulphurous Acid.—WADDINGTON (*Journ. Roy. Mic. Soc.*, 1883, p. 185) uses a saturated solution of sulphurous acid in alcohol for fixing infusoria. OVERTON (*Zeit. wiss. Mik.*, vii, 1890, p. 9) uses the vapours of an aqueous solution for fixing algæ.

CHAPTER V.

FIXING AND HARDENING AGENTS. CHLORIDES, ORGANIC ACIDS, AND OTHERS.

Chlorides.

69. Bichloride of Mercury (Corrosive Sublimate).—Corrosive sublimate is stated in the books to be soluble in about sixteen parts of cold and three of boiling distilled water. It will probably be found that the aqueous solution contains from 6 to 7 per cent. of the sublimate at the temperature of the laboratory. It is more soluble in alcohol (1:3) or in ether (1:4) than in water. Its solubility in all these menstrua is augmented by the addition of hydrochloric acid, ammonious chloride, or camphor. With sodium chloride it forms a more easily soluble double salt; hence sea-water may dissolve over 15 per cent.

The simple aqueous solutions sometimes deteriorate in even a short time through the formation of a pulverulent precipitate. Thinking that it may be due in part to ammonia derived from the air, I have lately been in the habit of adding a little nitric acid to my solutions, and certainly have found that they thus keep much better. In any case, for work in which it is desired to obtain as energetic a fixing action as possible, it is well to use only freshly made up solutions. And *distilled* water must *always* be employed for making up the solutions. The simple aqueous solution should give an acid reaction with litmus paper, whilst that made with strong sodium chloride solution is neutral.

For fixing, corrosive sublimate may be, and very frequently is, used pure; but in most cases a finer fixation will be obtained if it be *acidified with acetic acid*, say about 1 per cent. of the glacial acid. I find that a saturated solution in 5 per cent. glacial acetic acid is a very good formula for *marine* animals; for others I should take the acid weaker. Kaiser's solution consists of 10 g. sublimate, 3 g. glacial acetic acid, and 300 g. distilled water (from *Zeit. wiss. Mik.*, xi, p. 378). Van Beneden has used a saturated solution in 25 per cent.

acetic acid, and Lo Bianco (*Mitth. Zool. Stat. Neapel*, ix, 1890, p. 443) a mixture of 2 parts saturated solution with 1 part of 49 per cent. acetic acid.

It is sometimes advisable to take the most concentrated solution obtainable. The cold saturated aqueous solution will suffice in most cases; but for some very contractile forms (coral polypes, Planaria), a concentrated solution in warm or even boiling water should be employed. For Arthropoda *alcoholic* solutions are frequently indicated. Delicate objects, however, may require treatment with weak solutions.

Objects should in all cases be removed from the fixing bath as soon as fixed, that is, in other words, as soon as they are seen to have become opaque throughout, which is practically as soon as they are penetrated by the liquid. Small objects are fixed in a few minutes. I have found that a "salivary" gland of the larva of *Chironomus* is thoroughly fixed in three seconds.

Wash out with water or with alcohol. I consider alcohol almost always preferable. Alcohol of about 70 per cent. may be taken. The extraction of the sublimate is hastened by the addition of a little camphor to the alcohol. Or, much better (Mayer, *Intern. Monatsschr. Anat. Phys.*, iv, 1887, p. 43), a little tincture of iodine may be added to the liquid, either alcohol or water, used for washing, enough to make it of a good port-wine colour, and the liquid be changed until it no longer becomes discoloured by the objects. Apáthy (*Mikrotechnik*, p. 148) takes a 0·5 per cent. solution of iodine in strong alcohol, leaves the objects in it (suspended) until they have become of about the colour of the solution, and then washes for twenty-four hours in pure alcohol.

In *obstinate cases* solution of iodine in iodide of potassium may be taken. Mayer (*Zeit. wiss. Mik.*, xiv, 1897, p. 28) makes it by dissolving 5 grammes of iodide of potassium in 5 c.c. of distilled water and mixing this with a solution of 0·5 gramme of iodine in 45 c.c. of 90 per cent. alcohol, but seldom uses the mixture concentrated, merely adding as much of it as is required to the alcohol or water containing the objects. The iodine may be washed out in obstinate cases with magnesia water. Similarly Apáthy (*Mitth. Zool. Stat. Neapel*, xii, 1897, pp. 729, 730).

It has been objected to this process that iodine in potassic iodide precipitates corrosive sublimate instead of dissolving it. That is true, but the precipitate is soluble in excess of the precipitant.

The iodide of potassium process should be employed with care, for the iodide may not only dissolve out the free uncombined sublimate from the tissues, but also may partly redissolve the precipitated compounds formed by the sublimate with the albuminoids, etc., of the tissues (§ 27), thus so far undoing the work of fixation. Possibly the same may happen with iodine in water or weak alcohol, so that it may be well not to begin adding the iodine till the objects have been brought into fairly strong alcohol, 70 or 80 per cent.

It is important that the sublimate be *thoroughly* removed from the tissues, otherwise they become brittle, and will not stain so well. They will also become brittle if they are kept long in alcohol.

It may happen that if the extraction of the excess of sublimate from the tissues in bulk has been insufficient, crystals may form in the sections after they have been mounted in balsam. This may easily be prevented by treating the sections themselves with tincture of iodine for a quarter of an hour before mounting. Some workers hold that this does away with the necessity of treating the tissues in bulk with iodine, which is frequently a very long process. Thus, MANN (*Zeit. wiss. Mik.*, xi, 1894, p. 479) prefers treating the sections rather than the tissues in bulk, on the ground that the iodine makes them soft, so that they shrink on coming into paraffin. SCHAPER (*Anat. Anz.*, xiii, 1897, p. 463), however, has shown that neglect to extract the sublimate from the tissues in bulk may give birth to serious artefacts, which appear to arise during the imbedding process.

You may stain in any way you like. Carmine stains are peculiarly brilliant after sublimate.

It must be remembered that the solutions must not be touched with iron or steel, as these produce precipitates that may hurt the preparations. To manipulate the objects, wood, glass or platinum may be used; for dissecting them, hedgehog spines, or quill pens, or cactus spines.

When properly employed, sublimate is *for general work* undoubtedly a most useful fixing agent. It is applicable to most classes of objects. It is perhaps less applicable, in the pure form, to Arthropods, as it possesses no great power of penetrating chitin. For *cytological work* it is, according to my experience, not to be trusted, and only to be recommended where more precise fixing agents, such as solution of Flemming, are counter-indicated by reason of their lack of penetration, or the like. Amongst other defects it has that of frequently causing *very serious shrinkage* of cells.

70. Sublimate with Salt.—A solution containing 5 g. sublimate, 0·5 g. sodium chloride, and 100 c.c. water has been quoted as "solution of Gaule."

A one-half per cent. aqueous solution of sodium chloride saturated whilst hot with sublimate was for some years much in vogue through the recommendations of M. Heidenhain (*Festschrift f. Koelliker*, 1892, p. 109).

The addition of sodium chloride allows a stronger solution to be obtained than can be made with pure water (see last §), and also, it is stated, enhances the penetration of the sublimate. But against this advantage must be set the disadvantage that the fixation-precipitates (§ 27) formed by the double salt are (according to Spuler, *Encycl. mik. Technik.*, p. 1274) for the most part soluble in water, thus giving rise to imperfect preservation. It has been found by Paal (*l. c.*), that on adding sublimate dissolved in salt solution to a solution of white of egg, no visible precipitate is produced! It would seem to follow that salt should not be added to sublimate solutions, unless, indeed, they be corrected by the addition of a sufficient proportion of acetic acid.

71. Liquid of Lang (*Zool. Anzeiger*, 1878, i, p. 14).—For *Planaria.*—

Distilled water	100 parts by weight.
Chloride of sodium	6 to 10 parts.
Acetic acid.	6 to 8 ,,
Bichloride of mercury	3 to 12 ,,
(Alum, in some cases	½.)

72. Alcoholic Solutions.—Apáthy (*Mikrotechnik*, p. 111) writes that he thinks that "a solution of 3 to 4 grammes of sublimate and 0·5 gramme sodium chloride in 50 per cent. alcohol" (quantity not stated!) will prove to be "for most objects the best of fixatives for *general* purposes."

Ohlmacher (*Journ. Exper. Medicine*, ii, 6, 1897, p. 671) takes—

Absolute alcohol	80 parts.
Chloroform	15 ,,
Glacial acetic acid	5 ,,

Sublimate to saturation (about 20 per cent.).

"Ordinary pieces" of tissue are sufficiently fixed in fifteen to thirty minutes. Entire human cerebral hemispheres, subdivided by Meynert's section, take eighteen to twenty-four hours.

For liquids containing a much higher proportion of acetic acid, see *Acetic Alcohol*, §§ 91, 92, and see also § 74.

73. Aceton Solution.—Held (*Arch. Anat. Phys.*, Anat. Abth. 1897, p. 227), fixes nerve tissue in a 1 per cent.

solution of sublimate in 40 per cent. aceton, and washes out through increasingly-concentrated grades of aceton.

74. **Mercuro-nitric Mixtures.**—Frenzel (*Arch. mik. Anat.*, xxvi, 1885, p. 232) recommends a half-saturated solution of sublimate in 80 per cent. alcohol, to which is added nitric acid in the proportion of 1 drop to 1 c.c. or 2 c.c. Objects of the size of a pea to be fixed in it for five or ten minutes, then hardened in the same sublimate alcohol without the acid, and finally in 90 per cent. alcohol. It is said that the nitric acid renders after-treatment with iodine unnecessary.

Gilson's **Mixture.**—I am indebted to Prof. Gilson for kindly sending his latest formula (1895), which is as follows (I have simplified it by omitting one place of decimals) :

Nitric acid of 46° strength (this would be sp. gr. 1·456, or 80 per cent., nearly	15 c.c.
Glacial acetic acid . . .	4 ,,
Corrosive sublimate . . .	20 grms.
60 per cent. alcohol . . .	100 c.c.
Distilled water	880 ,,

When required *for marine animals* add a few crystals of iodine, which will prevent the formation of precipitates of sea salts. If in any case the preparations should show a granular precipitate, due probably to an abundance of phosphates in the tissues, the precipitate may be removed by washing with water containing a little tincture of iodine.

I have tried this mixture and find that it affords in general a faithful and delicate fixation, and gives to tissues an excellent consistency. Objects may remain in it for a considerable time without hurt. Tissues are left in a state very favourable for staining. The liquid has a high degree of penetration. A treatment for a few days with it will serve to remove the albumen from the ova of Batrachians. This liquid *may be recommended to beginners*, as it is very easy to work with. For some objects, as I found, the proportion of sublimate may be increased with advantage.

Kostanecki and Siedlecki (*Arch. mik. Anat.*, xlviii, 1896, p. 181) take a mixture of saturated sublimate solution and 3 per cent. nitric acid in equal parts, or a mixture of equal parts of sublimate solution, 3 per cent. nitric acid, and absolute alcohol, fix for twenty-four hours, and wash out in iodine-alcohol.

75. **Picro-sublimate Mixtures.**—LANG'S (*Zool. Auzeig.*, 1879, ii, p. 46).—A concentrated solution of corrosive sublimate in picro-sulphuric acid, to which has been added 5 per cent. of acetic acid.

RABL'S (*Zeit. wiss. Mik.*, xi, 1894, p. 165). Sublimate, saturated solution in water, 1 vol.; a similar solution of picric acid, 1 vol.; distilled water, 2 vols. Embryos may be left in it for twelve hours, washed for two hours in water, and brought into weak alcohol.

MANN'S (*op. cit.*, xi, 1895, p. 480).—1 per cent. of picric acid with or without 1 per cent. of tannin in a saturated solution of sublimate in normal salt solution.

The same author's **Alcoholic Picro-sublimate** (*Anat. Anz.*, viii, 1893, pp. 441—443) consists of absolute alcohol 100 c.c., picric acid 4 grms., sublimate 15 grms., tannin 6 to 8 grms. The tannin is added in order to prevent excessive hardening.

TELLYESNICZKY (*Arch. mik. Anat.*, lii, 1898, p. 237) says of Mann's tannin liquid, " its action is an entirely destructive one."

O. vom RATH'S **Picro-sublimate** (*Anat. Anz.*, xi, 1895, p. 268).—Cold saturated solution of picric acid, 1 part; hot saturated solution of sublimate, 1 part; glacial acetic acid, ½ to 1 per cent. Fix for several hours, and bring direct into alcohol.

The same author's **Picro-sublimate-osmic Mixture** (*loc. cit.*) consists of the above with the addition of 10 per cent. of 2 per cent. osmic acid solution.

See also § 119.

76. **Osmio-sublimate Mixtures.**—MANN'S (*Zeit. wiss. Mik.*, xi, 1894, p. 481) consists of a freshly-prepared mixture of equal parts of 1 per cent. osmic acid solution and saturated solution of sublimate in normal salt solution (for nerve-centres).

DRÜNER'S (*Jena. Zeit. Naturw.*, xxviii, 1894, p. 294) consists of 1 part of 1 per cent. osmic acid solution added to 20 parts of a solution of 5 per cent. each of sublimate and glacial acetic acid in water.

O. vom RATH'S, see last §.

77. Chromo-sublimate.—Lo Bianco (*Mitth. Zool. Stat. Neapel*, ix, 3, 1890, p. 443). Concentrated sublimate solution, 100 parts, 1 per cent. chromic acid, 50 parts.

Mann (*Verh. Anat. Ges.*, 12, 1898, p. 39) takes for nerve-cells equal parts of 5 per cent. sublimate and 5 per cent. chromic acid.

78. Sublimate and Bichromate.—Zenker's **Mixture** (*Münchener med. Wochenschr.*, 24, 1894, p. 534; quoted from Mercier, *Zeit. wiss. Mik.*, xi, 4, 1894, p. 471, where will be found minute instructions for using it). Five per cent. of sublimate and 5 per cent. of glacial acetic acid dissolved in solution of Müller. Fix for several hours, wash out with water, treat the tissues in bulk, or the sections with alcohol containing tincture of iodine.

If the objects be allowed to remain too long in the fluid there may be formed precipitates, which it is very difficult to remove. Spuler (*Encycl. mik. Technik.*, p. 1280) says that they may be avoided by removing the objects as soon as penetrated, and completing the hardening in liquid of Müller.

Dahlgren's modification, consisting of equal parts of Müller's solution and saturated sublimate solution with 5 per cent. of glacial acetic acid, gives fewer precipitates (Spuler, *l. c.*).

Helly (*Zeit. wiss. Mik.*, xx, 1904, p. 413) omits the acetic acid and adds, immediately before use, 5 per cent. of formol.

Zenker's liquid has been for some time very popular, and been found to preserve both nuclei and plasma without swelling or shrinkage, and to afford material that cuts well in paraffin and stains well with hæmatoxylin or carmine.

Lavdowsky's mixture has been given (§ 61) and Kultschizky's (§ 60).

79. Foà's **Mixture** (*Quart. Journ. Mic. Sci.*, 1895, p. 287).—Equal parts of saturated solution of sublimate in normal salt solution, and of liquid of Müller, or 5 per cent. solution of bichromate.

Very much like Zenker's mixture, with the acetic acid omitted, which appears to me to be certainly for most purposes a false step.

80. Bensley's **Mixture** (*Proc. Canadian Inst.*, v. 1897, p. 77; *Zeit. wiss. Mik.*, xvii, 1900, p. 233).—Equal parts of saturated solution of sublimate in 96 per cent. alcohol and 2 per cent. solution of bichromate in water. Wash out in 50 per cent. alcohol.

11. Platinum Chloride.—According to the two writers, Poll and Spuler, who treat of this subject in the *Encycl. Mik. Technik*, pp. 1142 to 1145, the "Platinchlorid" used and intended by the authors who have recommended this reagent is not the true platinic chloride, or tetrachloride, $PtCl_4$, but the compound H_2PtCl_6, that is, platinochloric, or hydrochloroplatinic acid, by custom called platinum chloride. It occurs as brown-red crystals, easily soluble in water and very deliquescent. For this reason it had better be stocked in the form of a 10 per cent. solution, kept in the dark (weak solutions—0·5 per cent.—may be kept in the light). Ten per cent solutions are found in commerce.

It appears that some authors have stated that they were using platinous chloride, $PtCl_2$, but that is not possible, as this salt is not soluble in water.

Rabl (to whom we owe the introduction of this agent) employed an aqueous solution of 1:300. The objects remained in it for 24 hours, and were then washed with water, hardened in alcohol, and sectioned.

The washing out with water should be very thoroughly done, days rather than hours being necessary. Well-washed preparations give good chromatin stains with the "basic" tar colours and with iron hæmatoxylin; but I find, as do others, that plasma-staining with the "acid" colours is rendered extremely difficult.

Rabl found it give better results (for the study of karyokinesis) than any other reagent except chromoformic acid (§ 44). It causes a certain shrinkage of the chromatin elements, a condition that renders the granules of Pfitzner and the longitudinal division of the elements very distinctly visible (see Rabl's paper in *Morph. Jahrb.*, Bd. x, 1884, p. 216).

It is now almost always employed in the form of mixtures. For these see §§ 49, 50, 53, *ante*, as well as the mixtures given under Picric acid and Formol and a formula of Rabl, § 599.

82. Palladium Chloride (F. E. Schulze, *Arch. mik. Anat.*, iii, 1867, p. 477).—This reagent was recommended by Schulze as a hardening agent, on account of a special faculty for penetrating organs rich in connective tissue that he attributed to it. It is an impregnation reagent, staining certain elements of tissues in various tones of brown. For the manner of employing it see the paper quoted. You wash out with water.

Cattaneo recommends it, used in solutions of 1·300, 1·600, or 1·800 strength, for from one to two minutes, as being the best of fixatives for Infusoria.

This salt is found in commerce in the solid state. To dissolve it, take 10 grammes of the salt, one litre of water, and four to six drops of hydrochloric acid. Solution will be effected in twenty-four hours.

Frenkel (*Anat. Anz.*, viii, 1893, p. 538) recommends for connective tissue a mixture of 15 parts 1 per cent. palladium chloride, 5 parts 2 per cent. osmic acid, and a few drops of acetic acid.

83. Iridium Chloride (Eisen, *Zeit. wiss. Mik.*, xiv, 1897, p. 195).—Solution of one half or one fifth per cent., acidified with 1 per cent. of glacial acetic acid.

When this formula was published I tried it on my usual test-object, the ovotestis of the snail, and obtained about the worst fixation I have ever seen. I have since tried it on the testis of *Triton*, and obtained much better results—chromatin good, cytoplasm not very bad, though very thinly fixed after an immersion of three days, plasma staining very bad.

84. Osmium Chloride (Eisen, *Journ. of Morph.*, xvii, 1900). Solution of $\frac{1}{2}$ to $\frac{1}{10}$ per cent. From specimens I have seen I should say it is useless.

85 Perchloride of Iron (Fol, *Zeit. wiss. Zool.*, xxxviii, 1883, p. 491, and *Lehrb. d. vergl. mik. Anat.*, p. 102).—Fol recommends 1 vol. of *Tinct. Ferri Perchlor.* B.P. diluted with 5 to 10 vols. of 70 per cent. alcohol.

The tincture diluted with 3 to 4 vols. of either alcohol or water has been recommended for fixing medullated nerve by Platner (*Zeit. wiss. Mik.*, vi, 1889, p. 187).

86. Chloride of Zinc is sometimes used for hardening brain (see Part II). Gilson (*La Cellule*, vi, 1890, p. 122) has used it as a fixative for the silk glands of Lepidoptera, as follows:

Glacial acetic acid	5 c.c.
Nitric acid of 46° (or 80 per cent. nearly)	5 ,,
Alcohol of 80 per cent.	100 ,,
Distilled water	300 ,,
Dry chloride of zinc	20 grammes.

87. Fluorides (Marpmann), see *Zeit. angew. Mik.*, v, 1899, p. 33, or *Journ. Roy. Mic. Soc.*, 1899, p. 456.

88. Iodine.—Iodine possesses considerable hardening properties, and a very high degree of penetration. Kent (*Manual of the Infusoria*, 1881,

p. 114; *Journ. Roy. Mic. Soc.* (N.S.), iii, 1883, p. 730) has found it to act in a manner almost identical with osmic acid for fixing Infusoria. Prepare a saturated solution of potassic iodide in distilled water, saturate this solution with iodine, filter, and dilute to a brown-sherry colour. A very small portion only of the fluid is to be added to that containing the Infusoria.

Or you may use LUGOL's solution, of which the formula is as follows:

Water	100 parts.
Iodide of potassium	6 ,,
Iodine	4 ,,

Iodide certainly kills cells very rapidly without deforming them. Personally I have found it very useful for the examination of spermatozoa.

Very small objects may be instantaneously fixed by means of **vapour of Iodine**. Crystals of iodine may be heated in a test-tube till the vapours are given off; then on inclining the tube the heavy vapours may be made to flow over the objects arranged on a slide. The slide should then be warmed to about 40° C. for one to three minutes in order to evaporate the iodine from the objects, which may then be mounted or otherwise treated as desired (OVERTON, *Zeit. wiss. Mik.*, vii, 1890, p. 14).

Iodine may be used in combination with alcohol for hardening, and render service through its great penetrating power. See the method of BETZ, in "Neurological methods."

Organic Acids, and other Agents.

89. Acetic Acid.—Flemming, who has made a special investigation of its action on *nuclei*, finds (*Zellsubstanz*, etc., p. 380) that the best strength is from 0·2 to 1 per cent. Strengths of 5 per cent. and more bring out the nuclein structures clearly at first, but after a time cause them to swell and become pale, which is not the case with the weaker strengths (*ibid.*, p. 103). Thanks to v. BENEDEN the *strong* acid has also become established as a valuable fixative of certain objects. It is particularly applicable to very contracticle objects, such as many Vermes, Cœlenterata, and Nudibranchs; it kills them with the utmost rapidity, *and has a tendency to leave them fixed in the state of extension.* The *modus operandi* is in general as follows:—Pour glacial acetic acid in liberal quantity over the organisms, leave them until they are penetrated by it—which should be in five or six minutes, as the strong acid is a highly penetrating reagent—and wash out in frequent changes of alcohol of gradually increasing strength. Some persons begin with 30 per cent. alcohol, but this appears to me rather weak, and I think 70 per cent. or at least 50 per cent. alcohol should be preferred.

Other energetic reagents may be combined with the glacial acetic acid if desired. Dr. LINDSAY JOHNSON (*in. litt.*) has found that one of the best fixatives for retina is a mixture of equal parts glacial acetic acid and 2 per cent. osmic acid. S. LO BIANCO adds to the "concentrated"* acid one tenth of a 1 per cent. solution of chromic acid. He finds that even this small proportion of chromic acid serves to counteract in a marked degree the softening action of the acetic acid.

It goes without saying that in v. Beneden's process the acetic acid does not play the part of a fixing agent *sensu stricto*, that is, an agent that *hardens* cells at the same time that it kills them. The *rationale* of the process is that the *acid kills* the tissues, whilst the *alcohol* comes in and *hardens* them sufficiently before they have had time to become deformed by the action of the acid.

Acetic acid, used alone, is only a fixative for a limited time. If its action be prolonged and not controlled by the action of some other agent, it becomes a *swelling* agent. Its function in mixtures is, besides that of killing, the valuable one of counteracting the shrinking action of the ingredients with which it is combined, and by its swelling action enhancing the penetration of the mixture; whilst by clarifying tissues it aids in the optical differentiation of their elements. Further (and this is most important), it acts in mixtures (as explained at length by FISCHER, see § 28) as an *acidifier*, serving to counteract any alkaline reaction of the tissues and to ensure that the precipitation of cell-contents, which constitute fixation, shall take place in an acid medium. This is a *sine quâ non* for the due fixation of nuclein. For these reasons it is, in all cases in which its presence is not absolutely counter-indicated (connective tissue, delicate calcareous structures, etc.), a most valuable ingredient, almost indispensable in fixing mixtures.

The proportions in which it should enter into mixtures in general seem to me to be from 0·5 per cent. to 5 per cent. of the glacial acid; higher strengths, such as 25 per cent. to 100 per cent., being only indicated in cases in which the highest possible penetration is the chief consideration.

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* MAYER, in the *Grundzüge*, explains that the acid referred to as "concentrated" by LO BIANCO in his *Metodi* (*Mitth. Zool. Stat. Neapel*, ix, 3, p. 435) is an acid of approximately 49 per cent. (sp. gr. 1·060).

Throughout this work, wherever acetic acid is mentioned, it is the *glacial* acid that is meant unless the contrary is stated.

All liquids containing a large proportion of this acid (*e.g.* §§ 90, 91) should only be allowed to act for a *very short time.*

90. Acetic Alcohol (CARNOY, *La Cellule*, iii, 1886, p. 6; and *ibid.*, 1887, p. 276; v. BENEDEN et NEYT, *Bull. Ac. Sci. Belg.*, xiv, 1887, p. 218; ZACHARIAS, *Anat. Anz.*, iii, 1888, pp. 24—27; v. GEHUCHTEN, *ibid.*, 8, p. 227.)—CARNOY has given two formulæ for this important reagent. The first is—

Glacial acetic acid	1 part.
Absolute alcohol	3 parts.

The second is—

Glacial acetic acid	1 part.
Absolute alcohol	6 parts.
Chloroform	3 ,,

The addition of chloroform is said to render the action of the mixture more rapid.

V. BENEDEN and NEYT take equal volumes of glacial acid and absolute alcohol.

ZACHARIAS takes—

Glacial acetic acid	1 part.
Absolute alcohol	4 parts.
Osmic acid	a few drops.

Acetic alcohol is one of the most penetrating and quickly acting fixatives known. It preserves both nuclei and cytoplasm, and admits of staining in any way that may be preferred. It was employed by all of the authors quoted for the study of karyokinesis in the ova of *Ascaris*—proverbially one of the most difficult objects to fix,—but from what I have seen of it I should say that it is applicable with advantage to many other objects. It has been found to give excellent results with central nervous tissue. You may wash out with alcohol and treat afterwards in any way that may be preferred (aqueous liquids being avoided as far as possible). But the sublimate liquid, next §, will probably be found in most cases superior.

91. Acetic Alcohol with Sublimate.—The following mixture, due to Gilson, was first published by Carnoy and Lebrun (*La Cellule,* xiii, 1, 1887, p. 68), and most highly recommended by them as superior for ova of *Ascaris* with the shell formed to the chloroform liquid of the last §. The addition of the sublimate serves to restrain the swelling action of the acetic acid, which is insufficiently done by the other ingredients.

Absolute alcohol	1 vol.
Glacial acetic acid . . .	1 ,,
Chloroform	1 ,,

Sublimate to saturation.

Isolated ova of *Ascaris,* even though furnished with a shell, are fixed in twenty-five to thirty seconds. Entire oviducts take about ten minutes. The liquid is therefore one of the most penetrating and rapidly acting of any, if not *the* most.

Wash out with alcohol until all traces of odour of the acetic acid have disappeared (I myself wash out with alcohol containing tincture of iodine). I consider this a very fine reagent.

For Ohlmacher's mixture see § 72.

92. Mingazzini's Mixture (*Ricerche Lab. Anat. Roma,* iii, 1893, p. 47).—Two vols. saturated aqueous solution of sublimate, one of absolute alcohol, and one of glacial acetic acid.

93. Formic Acid may be used *diluted* in the same way as acetic acid, (*supra,* § 89).

94. Trichlor-acetic Acid (Holmgren, *Anat. Hefte,* xviii, 1901, H. 2).—5 per cent. solution in water. Fix (nerve-cells) for 8 to 24 hours' wash out with alcohol.

95. Trichlor-lactic Acid (Holmgren, *Anat. Anz.,* xx, 1902, p. 435).—As the last.

96. Salicylic Acid (Heidenhain, *Arch. mik. Anat.,* liv, 1899, p. 186).—Saturated solution in one third alcohol. A trial has given me simply atrocious results.

97. Chloride and Acetate of Copper (*Ripart et Petit's Liquid*, Carnoy, *La Biologie Cellulaire*, p. 94).—

Camphor water (not saturated)	75 grammes.
Distilled water	75 ,,
Crystallised acetic acid	1 gramme.
Acetate of copper	0·30 ,,
Chlorideof copper	0·30 ,,

This is a very moderate and delicate fixative. I consider that it has not sufficient hardening power for objects that are intended to be dehydrated and mounted in balsam, but is extremely useful for objects that are to be studied in as fresh a state as possible in aqueous media. Objects fixed in it stain instantaneously and perfectly with methyl green. Osmic acid may be added to the liquid to increase the fixing action. For *cytological researches* this is a most valuable medium, see § 652.

98. Acetate of Uranium (Schenk, *Mitth. Embryol. Inst. Wien*, 1882, p. 95; cf. Gilson, *La Cellule*, i, 1885, p. 141).—This reagent has a mild fixing action, and a high degree of penetration, which may make it useful for Arthropoda. It may be combined with methyl green, which it does not precipitate.

For **Acetate of Lead** see "Neurological Methods," Hardening.

99. Picric Acid.—Picric acid in pure aqueous solution should always be employed in the form of a *strong* solution. (That is to say, strong solutions must always be employed when it is desired to make sections or other preparations of tissues with the elements *in situ*, as weak solutions macerate; but for dissociation preparations or the fixation of isolated cells, weak solutions may be taken. Flemming finds that the fixation of nuclear figures is equally good with strong or weak solutions.) The saturated solution is the one most employed. (One part of picric acid dissolves in about 86 parts of water at 15° C.;* in hot water it is very much more soluble.) Objects should remain in it for from a few seconds to twenty-four hours, according to their size. For Infusoria one to at most two minutes will suffice, whilst objects of a thickness of several millimetres require from three to six hours' immersion.

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* Benedikt and Knecht, *Chemistry of the Coal-tar Colours*, p. 214.

Picric acid should *always be washed out with alcohol*, as water is hurtful to tissues that have been prepared in it. For the same reason during all remaining stages of treatment, water should be avoided; staining should be performed by means of alcoholic solutions, the only exceptions to this rule being in favour of methyl green, and some few other aqueous stains that are themselves weak hardening agents, such as hæmalum, carmalum, etc.

Washing out is facilitated by heat, the extraction being about twice as rapid at 40° C. as at the normal temperature (Fol).

It has been found by Jelinek (*Zeit. wiss. Mik.*, xi, 1894, p. 242) that the extraction is greatly quickened by the addition of a base to the wash-alcohol. He recommends carbonate of lithia. A few drops of a saturated solution of the salt in water are added to the alcohol; a slight precipitate is formed. The objects are put into the turbid alcohol, which becomes clear and yellow in proportion as the picrin is extracted. Further quantities of carbonate are added from time to time until the colour has been entirely extracted from the tissues.

Tissues fixed in picric acid can be perfectly stained in any stain. It is not generally necessary to remove the picric acid by washing out before staining. Mayer's paracarmine, Grenacher's alcoholic borax-carmine, or Mayer's hæmacalcium may be recommended for entire objects.

The most important property of picric acid is its great penetration. This renders it peculiarly suitable for the preparation of chitinous structures. For such objects alcohol of 70 per cent. to 90 per cent. should be taken for washing out, and staining should be done by means of Mayer's cochineal or hæmacalcium.

100. Picro-acetic Acid.—Boveri (*Zellenstudien*, 1, 1887, p. 11) dilutes a concentrated aqueous solution of picric acid with two volumes of water and adds 1 per cent. of acetic acid. According to my experience, the results are most miserable.

101. Picro-sulphuric Acid (Kleinenberg, *Quart. Journ. Mic. Sci.*, April, 1879, p. 208; Mayer, *Mitth. Zool. Stat. Neapel*, ii, 1880, p. 2).—Mayer takes distilled water, 100 vols.; sulphuric acid, 2 vols.; picric acid, as much as will dissolve.

Liquid of Kleinenberg is made by diluting the concentrated picro-sulphuric acid prepared as above with three times its volume of water.

Of these two formulæ the one formerly most employed is that given by Kleinenberg—the dilute mixture; undiluted picro-sulphuric acid being reserved for objects requiring special treatment, chiefly Arthropods. I hold that the concentrated solution is generally preferable. *This particularly applies to marine organisms.*

Wash out with successive alcohols, beginning with 70 per cent., never with water.

Warm alcohol extracts the acid much more quickly than cold, without which *weeks* may be required to fully remove the acid from chitinous structures.

This liquid, once the classical fixative, is now almost entirely abandoned, I think rightly, as its fixing qualities are at the best only third-rate. For Arthropoda it may still be useful, on account of its great power of penetrating chitin. For a fuller account *see previous editions.*

102. Picro-nitric Acid (Mayer, *Mitth. Zool. Stat. Neapel*, 1881, p. 5).—

Water	100 vols.
Nitric acid (of 25 per cent. N_2O_5) . .	5 ,,

Picric acid, as much as will dissolve.

The properties of this fluid are very similar to those of picro-sulphuric acid, with the advantage of avoiding the formation of gypsum crystals, and the disadvantage that it is much more difficult to soak out of the tissues. The process of Jelinek, § 99, may be useful here. Mayer states that with eggs containing a large amount of yolk material, like those of Palinurus, it gives better results than nitric, picric, or picro-sulphuric acid. I myself consider it distinctly superior to picro-sulphuric.

103. Picro-hydrochloric Acid (Mayer, *ibid*).—

Water	100 vols.
Hydrochloric acid (of 25 per cent. HCl) .	8 ,,

Picric acid, as much as will dissolve.

104. Picro-chromic Acid (Fol, *Lehrb.*, p. 100).—

Picric acid, sol. sat. in water . .	10 vols.
1 per cent. chromic acid solution . .	25 ,,
Water	65 ,,

I have seen Fol's formula, with the addition of a trace of acetic acid, quoted as "liquid of Haensel"—I know not with what justification.

Lo Bianco takes equal parts of picro-sulphuric acid and chromic acid of 1 per cent.

105. Picro-nitro-chromic Acid (RAWLITZ, *Leitfaden*, 1895, p. 24).—One part of picro-nitric acid, and four parts 1 per cent. chromic acid. Wash out in 70 per cent. alcohol.

106. Picro-osmic Acid.—FLEMMING (*Zells. Kern u. Zellth.*, p. 381) has experimented with mixtures made by substituting picric for chromic acid in the chromo-osmic mixtures (*ante*, §§ 46 and 47). The results, he says, are identical, so far as regards the fixation of nuclei. He may be right as regards the nuclei; but the fixation of cytoplasm is in my preparations decidedly inferior.

O VOM RATH (*Anat. Anz.*, xi, 1895, p. 289) adds to 200 c.c. of saturated aqueous solution of picric acid, 12 c.c. of 2 per cent. solution of osmic acid, and 2 c.c. of glacial acetic acid.

107. Picro-nitro-osmic Acid (RAWITZ, *Leitfaden*, p. 24).—Picro-nitric acid, 6 vols.; 2 per cent. osmic acid, 1 vol. Fix for ½ to 3 hours. Transfer direct to 70 per cent. alcohol. The mixture keeps well.

108. Picro-platinic and Picro-platin-osmic Mixtures.—O VOM RATH (*loc. cit.*, § 106, pp. 282, 285) makes a picro-platinic mixture with 200 c.c. saturated aqueous solution of picric acid, 1 g. of platinic chloride (dissolved in 10 c.c. of water), and 2 c.c. of glacial acetic acid.

The picro-platin-osmic mixture, which is, in my opinion, much superior, is made by adding to the foregoing 25 c.c. of 2 per cent. osmic acid.

109. Picric Alcohol (GAGE, *Proc. Amer. Soc. Micr.*, 1890, p. 120).—Alcohol (95 per cent.), 250 parts; water, 250 parts; picric acid, 1 part.

110. Other Picric Mixtures.—See § 75, also §§ 117 to 119.

Other Fixing and Hardening Agents.

111. Alcohol.—For *fixing* it is generally held that only two grades of alcohol should be employed—very weak alcohol on the one hand, and absolute on the other. Absolute alcohol is held to rank as a fixing agent because it is said to kill and harden with such rapidity that structures have not time to get deformed in the process by the energetic dehydration that unavoidably takes place. Dilute alcohol is held to rank as a fixing agent in virtue of being of such a strength as to possess a sufficiently energetic coagulating action and yet contain enough water to have but a feeble and innocuous dehydrating action. The intermediate grades, it is held, do not realise these conditions, and therefore should not be employed alone for fixing. But they may be very useful in combination with other fixing agents (such as corrosive sublimate or nitric acid) by greatly enhancing their penetrating power; 70 per cent is a good grade for this purpose.

TELLYESNICZKY (*Arch. Mik. Anat.* lii, 1898, p. 219) disagrees with the doctrine of the supposed importance of the grades used, finding them all equally bad. He finds no difference at all between the action of absolute alcohol and that of 96 per cent. or that of 70 per cent. They all cause a remarkable amount of shrinkage, and probably a notable amount of solution of cell-constituents.

Alcohol is an easily *oxidisable* substance. Chromic acid, for instance, easily oxidises it, first into aldehyde, and then into acetic acid. It follows that alcohol should not be combined in mixtures with oxidising agents of notable energy, if it be desired to preserve it *as* alcohol in the mixture. Further, alcohol is a *reducing* agent, and therefore should not be combined with easily reducible substances. These remarks particularly apply to chromic acid, see §§ 41, 42, 52.

For *fixing*, alcohol is a very third-class reagent, only to be used alone where better ones cannot be conveniently employed, though it enters as a most important ingredient into many *mixtures*, in which it serves to enhance the power of penetration. For *hardening* it is a very important one. When used alone it is indeed inferior as a hardening agent to most of the reagents discussed above; but when judiciously employed to complete the action of a good fixing agent, it renders most valuable services. 90 to 95 per cent. is the most generally useful strength. Weaker alcohol, down to 70 per cent., is often indicated. Absolute alcohol is seldom advisable. You ought to begin with weak, and proceed gradually to stronger, alcohol. Large quantities of alcohol should be taken. The alcohol should be frequently changed, or the tissue should be suspended near the top of the alcohol, in order to have the tissue constantly surrounded with pure spirit (the water and colloid matters extracted from the tissue falling to the bottom of the vessel). Many weeks may be necessary for hardening large specimens.

Small pieces of permeable tissue, such as mucous membrane, may be sufficiently hardened in twenty-four hours.

112. Absolute Alcohol.—This is sometimes valuable on account of its great penetrating power, being, indeed, one of the most penetrating of known fixing agents. Mayer finds that boiling absolute alcohol is often the only means of killing certain Arthropoda rapidly enough to avoid maceration brought about by the slowness of penetration of common cold alcohol (especially in the case of Tracheata).

It is important to employ for fixing a very large proportion of alcohol. Alum-carmine is a good stain for small specimens so fixed. For preservation, the object should be put into a weaker alcohol, 90 per cent. or less.

As to the supposed superiority of absolute alcohol over ordinary strong alcohol, see last § ; and amongst authors upholding its superiority, see besides RANVIER, MAYER (*Mitth. Zool. Stat. Neapel*, ii, 1880, p. 7) ; BRÜEL (*Zool. Jahrb., Abth. Morph.*, x, 1897, p. 569) ; and VAN REES (*ibid.*, iii, 1888, p. 10).

Absolute alcohol is found in commerce. It is a product that it is almost impossible to preserve in use, on account of the rapidity with which it hydrates on exposure to air. Fol recommends that a little quicklime be kept in it. This absorbs part at least of the moisture drawn by the alcohol from the air, and has the further advantage of neutralising the acid that is frequently present in commercial alcohol.

Another plan that I have seen recommended is to suspend strips of gelatin in it. It is stated that by this means ordinary alcohol may be rendered absolute. But then it is probably also rendered very acid thereby.

Ranvier adopts the following plan for preparing an alcohol absolute enough for all practical purposes. Strong (95 per cent.) alcohol is treated with calcined cupric sulphate, with which it is shaken up and allowed to remain for a day or two. It is then decanted and treated with fresh cupric sulphate, and the operation is repeated until the fresh cupric sulphate no longer becomes conspicuously blue on contact with the alcohol; or until, on a drop of the alcohol being mixed with a drop of turpentine, no particles of water can be seen in it under the microscope. The cupric sulphate is prepared by calcining common blue vitriol in a porcelain capsule over a spirit lamp or gas burner until it becomes white, and then reducing it to powder (see *Proc. Acad. Nat. Sci. Philad.*, 1884, p. 27 ; *Journ. Roy. Mic. Soc.*, 1884, pp. 322 and 984).

Test for the presence of water (YVON, *C. R. Acad. Sci.*, 1897, p. 1181).—Add coarsely powdered calcium carbide; the merest trace of water will cause an evolution of acetylene gas, and on agitation the alcohol will become turbid. (Calcium carbide may also serve for preparing absolute alcohol, but the alcohol must then be subsequently distilled twice, the second time over calcined cupric sulphate.)

113. One-third Alcohol.—The grade of weak alcohol that is generally held to be most useful for fixing is one-third alcohol, or RANVIER'S ALCOHOL. It consists *of two parts of water and one part of alcohol of* 90 *per cent.* (and *not* of absolute alcohol, as was stated by an oversight in the first edition—an error which I have seen copied in more than one place). See the *Traité Technique* of Ranvier, p. 241, *et passim.*

Objects may be left for twenty-four hours in this alcohol; not more, unless there be no reason for avoiding *maceration,* which will generally occur after that time. You may conveniently stain with picro-carmine, alum-carmine, or methyl green.

This reagent is a very mild fixative. Its hardening action is so slight that it is not at all indicated for the fixing of objects that are intended to be sectioned. Its chief use is for extemporaneous and dissociation preparations.

114. Acid Alcohol (MAYER, *Mitth. Zool. Stat. Neapel,* ii, 1881, p. 7).—To 97 vols. of 90 per cent. alcohol add 3 vols. pure hydrochloric (or nitric) acid. Wash out with 90 per cent. alcohol.

The use of this mixture is for the *preparation of coarse objects* it is intended to preserve in alcohol. The object of the acid is to prevent both that glueing together of organs by the perivisceral liquid, which is often brought about by the coagulating action of pure alcohol, and the precipitation on the surface of organs of the salts contained in sea-water, which is a hindrance not only to the penetration of the alcohol, but also to subsequent staining.

LO BIANCO (*Mitth. Zool. Stat. Neapel,* ix, 1890, p. 443) takes 50 per cent. alcohol with 5 per cent. of hydrochloric acid.

115. Formaldehyde, Formic Aldehyde, Methyl Aldehyde (Formol, Formalin, Formalose).—Formaldehyde is the chemical name of the gaseous compound HCOH, obtained by the oxidation of methyl-alcohol. "Formalin" is the commercial name given by SCHERING & Co. to a 40 per cent. solution of this substance in water. "Formol" is the commercial name given to the same solution by MEISTER, LUCIUS, & BRÜNING. And "Formalose" is the name for the same solution adopted by an American firm. (These solutions may now be obtained from dealers in photographic chemicals.) As I have before pointed out (*Anat. Anz.*, xi, 8, 1895, p. 255), the already extensive literature which treats of the anatomical uses of formaldehyde is much confused by inaccurate use of these terms; many writers use them indiscriminately. It is frequently impossible to discover from the statements of an author whether he means such or such a percentage of formaldehyde, or such or such a percentage of the commercial 40 per cent. solution employed by him, the one being of course two and a half times stronger than the other. All that can be said is, that the majority of authors seem to quote in percentages of the commercial solutions. I think it must be admitted that the proper way of stating the strength of these solutions is either to state them in terms of formaldeyhde, and say so, or to say "formol, or formalin, diluted with so many volumes of water."

Solutions of formaldehyde sometimes decompose partially or entirely, with formation of a white deposit of paraformaldehyde. FISH says that to avoid this the solutions should be kept in darkened bottles in the cool. The vapour of formaldehyde has a very irritating action on the conjunctiva and mucous membranes, but the effect is transitory, not so injurious as that of osmic acid. It is well not to soil the fingers with the solutions, as formaldehyde hardens the living skin very rapidly.

The solutions almost always have an acid reaction, due to the presence of formic acid; but that is, as a rule, rather an advantage.

It was discovered independently by F. BLUM (*Zeit. wiss. Mik.*, x, 1893, p. 314) and by HERMANN (*Anat. Anz.*, ix, 1893, p. 112) that formaldehyde possesses certain hardening and preservative properties.

BLUM employed formol diluted with ten volumes of water (containing rather less than 4 per cent. of formaldehyde). He found this solution to penetrate rapidly, and to harden *voluminous organs* such as liver, kidney, brain, *more rapidly than alcohol*, and that sections were well preserved and susceptible of good staining.

HERMANN used a solution containing 0·5 to 1 per cent. of "formalin" (the context shows that 1 per cent of *formaldehyde* is what is meant, the solution being made by diluting

Schering's formalin with forty volumes of water). He found it harden very rapidly, with the remarkable result that the hardened organs preserve *approximately the transparency of life*, and that *pigments are not discoloured*. Since that time formaldehyde has been largely used—in some cases misused—for the preparation and preservation of *museum specimens*, for which purpose it is in some respects superior to alcohol (for the employment of formaldehyde in museum work, see BLUM, *Zool. Anz.*, xvi, 1893, p. 450, and *Verh. Anat. Ges.*, 8 Vers., 1895, p. 236; KAISERLING *Arch. path. Anat.*, cxlvii, 1897, p. 396; MELNIKOFF-RASVEDENKOFF, *Compt. Rend.*, cxxiv, 1897, p. 238). Signs are, however, not wanting that it is by no means the elixir that has been supposed, and that it is a great mistake to imagine that it can take the place of alcohol as a *definitive* preservative of anatomical or museum specimens.

It was said above that formaldehyde possesses *certain* hardening and preservative qualities, the limitation intended being that it does not harden and preserve everything. It hardens gelatine, for instance, and certain albuminoids; but others, on the contrary, are not hardened by it, but rendered, on the contrary, more soluble than they are naturally. For some theoretical considerations concerning its action on tissues, see F. BLUM, in *Anat. Anz.*, xi, 1896, p. 718; BENEDECENTI, in *Arch. Anat. u. Phys.*, Abth., 1897, p. 219; GEROTA, in *Intern. Monatschr. Anat.*, xiii, 1896, p. 108; *Zeit. wiss. Mik.*, xiii, p. 311; SJÖBRING in *Anat. Anz.*, xvii, 1900, p. 274; and BLUM, in *Encycl. Mik. Technik.*, p. 393. It seems to be generally admitted that this action consists in the formation of methylene compounds with the substances of the tissues.

On account of its hardening properties it is much used as a *fixing agent*. Owing to the confusion in terminology above referred to, it is not possible to give precise instructions as to the strengths that have been employed by the different authors for this purpose. All that can be said is that they will almost certainly be bound to lie between the limits of those indicated by BLUM and HERMANN, that is to say between 0·5 per cent. and 4 per cent. *if the formaldehyde be used pure* (*i.e.* without admixture of any other fixing agent), and the latter is certainly by far the most usually employed strength. Only one writer (HOYER, jun., *Anat. Anz.*, ix, 1894, Ergänzungsheft, p. 236; *Zeit. wiss. Mik.*, xii, 1895, p. 28) appears to have used concentrated solutions. He states that with such solutions tissues are better preserved than with weak ones, even better preserved than with corrosive sublimate.

There is certainly some mistake here. I find that preparations fixed in 13·3 per cent. formaldehyde (formol with two volumes of water) have the cells enormously over-fixed. Experimenting further with weak solutions containing from 2 per cent. to 4 per cent. of formaldehyde, I have found that like the stronger solution mentioned above, these too give a homogeneous colloid appearance to protoplasm, and have at the same time a marked *swelling and vacuolating action.* With the 2 per cent. solution the vacuolation is enormous. I have concluded that, *used pure,* formaldehyde is not at all suitable as a fixing agent for cytological work.

Formol-fixed material is generally transferred direct to alcohol (of 50 per cent. and upwards), though some anatomists employ watery media for certain purposes.

To sum up, I feel convinced that neither strong nor weak should formaldehyde be employed *pure* for fixing, but that in combination with other substances it may play a valuable part in fixing *mixtures.*

It should be noted by those who desire to experiment with such mixtures, that formaldehyde is a powerful reducing agent, and therefore *incompatible* with such reagents as chromic acid or osmic acid and the like, which it very rapidly decomposes.

For *hardening,* formaldehyde is now much used, both for nervous tissue (see *Neurological Methods*) and for other tissues.

HERMANN (*loc. cit. supra*) found that such a large organ as a calf's heart was entirely hardened by a 0·5 to 1 per cent. sol. in twelve to twenty-four hours. *Entire eyes* are so hardened in the 1 per cent. solution in twenty-four hours that they may be cut in two with a sharp knife, like an apple. Hermann found this disadvantage, that tissues hardened in formaldehyde solution suffer when they are put into alcohol for the purpose of dehydration. The paper in question contains interesting observations on the property formaldehyde has of preserving the natural colours and transparent and lifelike aspect of tissues.

BLUM (*Anat. Anz.*, ix, 1894, p. 229), recapitulating, says that very voluminous pieces of material are hardened *quickly* and without shrinkage. The tissues stain well. Cells, he says, and nuclei preserve their forms; karyokinetic figures are fixed. Mucin is not precipitated, but remains transparent; fat is not dissolved. Micro-organisms retain their specific staining reactions.

As to the *degree and kind* of hardening obtained by formaldehyde the authors are not so explicit as could be wished. As far as I can see myself, the hardening obtained is gentle and tough, giving an elastic and not a brittle consistency. It varies greatly with different tissues.

For prolonged hardening, considerable volumes of liquid should be taken, and the liquid should be renewed from time to time; for the formaldehyde fixes itself on the tissues with which it comes in contact, deserting the solution, which thus becomes progressively weaker. The specimens should be suspended in the liquid or otherwise isolated from contact with the containing vessel.

It is one of the advantages of formol that it leaves tissues amenable to almost any kind of staining or impregnation.

Formaldehyde, being a powerful reducing agent, may be employed for the *reduction* of gold and silver impregnations. I have been using it myself for reducing gold impregnations, and up to the present like it better than any other agent I have tried for that purpose.

It is also a powerful *antiseptic*, and may be found very useful for effecting the preservation of *staining solutions*, with some of which it acts as a *mordant*. It is said to harden celloidin as well as gelatin, and to be useful for celloidin-imbedding (BLUM, *Anat. Anz.*, xi, 1896, p. 724).

116. Alcoholic Formol (LAVDOWSKY, *Anat. Hefte*, iv, 1894, p. 361.) Water 40 parts, 95 per cent. alcohol 20, formol 6, acetic acid 1; or water 30, alcohol 15, formol 5, acetic acid 1.

117. Picro-Formol.—P. BOUIN (*Phénomènes cytologiques anormaux dans L'Histogenèse*, etc., Nancy, 1897, p. 19) recommends—

Picric acid, saturated aqueous sol.	75 parts.
Formol	25 „
Acetic acid	5 „

Wash out with alcohol.

This formula has given me some of the finest preparations I have ever seen. The penetration is great, the fixation equable, delicate detail well preserved, staining qualities admirable, especially with iron-hematoxylin and Saürefuchsin.

The formulæ of GRAF (*State Hosp. Bull. New York*, 1897; *Journ. Roy. Mic. Soc.*, 1898, p. 492) are in my view too weak, and suffer by the omission of the acetic acid.

118. Picro-platinic Formol (M. and P. BOUIN, *Bibl. Anat.*, 1898, f. 2, p. 2).—

Platinum chloride, 1 per cent. sol.	20 parts.
Picric acid, saturated sol.	20 „
Formol	10 „
Formic or acetic acid	5 „

The platinum will certainly be found a hindrance to plasma staining.

119. Picro-sublimate Formol (M. and P. BOUIN, *loc. cit.*) A similar mixture, in which sublimate of 1 per cent. is substituted for the platinum chloride. This mixture is favourable for all sorts of staining.

SPULER (*Encycl. mik. Technik.*, p. 1280) recommends adding to sublimate (of 3 per cent. or more) 1 per cent. of glacial acetic acid and 10 per cent. of formol.

The formulæ of MANN (*Verh. Anat. Ges.*, 1898, p. 39), and BORÜN (*Arch. Ital. Biol.*, xvii, 1900, p. 211) have the defect of containing no acetic acid.

MAYER (*Grundzüge*, p. 67) finds mixtures of this sort quickly precipitate.

120. Formol-Müller.—This is the name given by ORTH (*Berl. Klin. Wochenschr.*, 1896, No. 13) to a mixture of 1 part of formol with 10 of liquid of Müller (§ 57). It should be freshly made up. Fix for three hours in the stove, or twelve at normal temperature, wash out with running water. Much used, especially for nervous tissues.

MOELLER (*Zeit. wiss. Zool.*, lxvi, 1899, p. 85) takes 1 vol. of formol and 4 of 3 per cent. bichromate (for the intestine of mammals).

121. Chromo-formol.—Various mixtures of formol with chromic acid have been recommended; they are evidently irrational (see § 115).

CHAPTER VI.

DE-ALCOHOLISATION AND CLEARING AGENTS.

122. Introduction.—*De-alcoholisation agents* are liquids employed for the purpose of getting rid of the alcohol which has been employed for dehydrating tissues (§ 3), and facilitating the penetration of the paraffin used for imbedding, or the balsam or other resinous medium in which preparations are, in most cases, finally mounted. Hence all of them must be capable of expelling alcohol from tissues, and must be at the same time solvents of Canada balsam and the other resinous mounting media. The majority of them are essential oils.

Clearing agents are liquids whose function it is to make microscopic preparations transparent by penetrating amongst the highly refracting elements of which the tissues are composed, the clearing liquids themselves having an index of refraction superior, or equal, or, at all events, not greatly inferior to that of the tissues to be cleared. Hence all clearing agents are liquids of high index of refraction.

The majority of de-alcoholisation agents being also liquids of high refraction, it follows that they serve at the same time for de-alcoholisation and for clearing; and in consequence it has come about that de-alcoholisation agents are generally spoken of as clearing agents. But that practice is not strictly correct, for not all clearing agents are solvents of the resins, and not all de-alcoholisation agents can serve as clearers. For instance, glycerine is a clearing agent, but cannot be used to prepare objects for paraffin or for balsam, because it is not miscible with either of them. And chloroform is an admirable de-alcoholisation agent and admirable precursor of paraffin or balsam, but can hardly be utilised as a clearer—*i.e.* for the purpose of obtaining the transparency required for examination—on account of its volatility, which precludes its use as an examination medium. I shall, however, still in many cases continue to use the term "clearing" to signify "de-alcoholising," for the sake of brevity.

Of course clearing media can serve as *Examination Media,* if not too volatile.

Neelsen and Schiefferdecker (*Arch. Anat. Phys.,* 1882, p. 206) examined a large series of ethereal oils (prepared by Schimmel and Co., Leipzig), with the object of finding a not too expensive substance that should combine the properties of clearing quickly alcohol preparations, *not* dissolving out anilin colours, clearing celloidin without dissolving it, not evaporating too quickly, and not having a too disagreeable smell.

Of these, the following three fulfil the conditions :—*Cedar-wood, Origanum, Sandal-wood.*

To these should be added the others recommended in the following paragraphs.

See also the paper of Jordan (*Zeit. wiss. Mik.,* xv, 1898, p. 50), which has special reference to the behaviour of some essential oils towards celloidin.

123. The Practice of De-alcoholisation or Clearing.—The old plan was to take the object out of the alcohol and float it on the surface of the de-alcoholising or clearing medium in a watch-glass. This plan was faulty, because the alcohol escapes from the surface of the object into the air quicker (in most instances) than the de-alcoholising or clearing agent can get into it; hence the object must shrink. To avoid or lessen this cause of shrinkage, the operation is now generally done by the method suggested by Mayer and Giesbrecht, which consists in putting the clearing medium *under* the alcohol containing the object, as described § 5. The objects should not be considered to be perfectly penetrated by the clearing medium until the wavy refraction-lines caused by the mixture of the two liquids at their surface have ceased to form. They may then be removed by means of a pipette, or the supernatant alcohol drawn off and the preparations allowed to remain until wanted.

The penetration of all clearing media may be hastened by using them warm.

It frequently happens that the essential oil with which objects are being treated in a watch-glass or on a slide becomes cloudy after a short time, and fails to clear the tissues. This is owing to a combination between the essential oil and moisture, derived, I think, rather from the air than from the objects themselves. The cloudiness can usually be removed by warming (as pointed out by HATCHETT JACKSON, *Zool. Anzeig.*, 1889, p. 630), but this remedy is not always successful, for in certain moist states of the atmosphere the cloudiness will persist, notwithstanding continued warming. It is for this reason that I advise that clearing be done, whenever possible, in shallow well-corked tubes, under which conditions the phenomenon rarely occurs. In any case, be careful not to breathe on the liquid.

124. Refractive Indices of Clearing Agents.—The following short list, extracted from BEHRENS' *Tabellen zum Gebrauch bei mikroskopischen Arbeiten*, Braunschweig, 1892, p. 42, and other sources, may be useful as a guide to the *optical effects* of various clearing media. The greatest transparency is obtained when the refraction of the medium is the same as that of the tissue elements. Media having a lower index than that of the tissues give diminished transparency, but greater boldness of detail. Media having a higher index than that of the tissues give great transparency, but diminished visibility of (unstained) details (see § 450). Now the index of refraction of most tissue elements, after fixation and dehydration, is somewhat higher than that of Canada balsam; so that media of the greatest clearing power must be looked for amongst reagents having an index superior to that of balsam, whilst for enhanced visibility of detail we must employ less refractive media, such as castor oil, glycerin, or water.

The figures given below must be accepted with some caution, on account of the variability of samples. The figures given for balsam refer evidently to the resin in the solid state and not to the solutions used for mounting, which are certainly much lower according to the lower index of the solvent.

Air	1·000
Methyl alcohol	1·323
Distilled water	1·336
Sea water	1·343
Solution of white of egg	1·350
Absolute alcohol	1·367
Acetate of potash, saturated aqueous sol.	1·370
Glycerine with an equal quantity of water	1·397
Chloride of calcium, 90 per cent. in water	1·411
Glycerine, Price's	1·460
Oil of bergamot	1·464
Paraffinum liquidum	1·471
Olive oil	1·473
Oil of turpentine	1·473
Glycerine, "concentrated"	1·473
Castor oil	1·490
Xylol	1·497
Cedar-wood oil, not thickened	1·510
Crown glass	1·518
Cedar-wood oil, thickened	1·520
Oil of lemons	1·527
Oil of cloves	1·533
Canada balsam (solid)	1·535
Creasote	1·538
Carbolic acid	1·549
Oil of anise seed	1·557
Anilin oil	1·580
Oil of cinnamon (or cassia)	1·619
Sulphide of carbon	1·630
Tolu balsam	1·640
Monobromide of naphthalin	1·660
Solution of sulphur in sulphide of carbon	1·750

See also § § 440 and 448.

It will be seen that cedar oil has nearly the index of crown glass (this is true of the oil in the thick state to which it is brought by exposure to the air—not of the new, thin oil, which is less highly refractive); it therefore clears to about the same extent as Canada balsam. Clove oil has a much higher index, and therefore clears more than balsam; cinnamon oil higher still. Turpentine and bergamot oil have much lower indices, and therefore clear less. Xylol is a good medium for the cursory examination of paraffin sections, provided that the examination does not last long enough to allow of its evaporating.

125. Choice of a De-alcoholisation or Clearing Agent.—I advise the beginner to keep on his table the following:—Oil of cedar, for general use and for preparing objects for imbedding in paraffin; clove oil, for making minute dissections in (§ 127), and for much work with safranin, etc.; oil of bergamot, which will clear from 90 per cent. alcohol, and which does not extract coal-tar colours; carbolic acid, for rapidly clearing very imperfectly dehydrated objects.

For special clearers for *celloidin sections* see § 179.

126. Cedar Oil (Neelsen and Schiefferdecker, *loc. cit.*,

§ 122).—Clears readily tissues in 95 per cent. alcohol without shrinkage ; does not extract anilin colours. Celloidin sections are cleared in five to six hours.

The observer should be careful as to the quality of the cedar oil he obtains. I have examined the clearing properties of a sample obtained from the celebrated firm of Rousseau, Paris. This sample was absolutely colourless. It *totally* failed to clear absolute alcohol objects after many days.

Cedar oil is very penetrating, and for this and other reasons is, in my experience, the very best of all media for preparing objects for paraffin imbedding. I find it to be *less hurtful to cells* and delicate tissue-structures than any other medium known to me. If it should become milky through keeping, filter.

127. Clove Oil.—Samples of clove oil of very different shades of colour are met with in commerce. It is frequently recommended that only the paler sorts should be employed in histology. Doubtless it is, in general, best to use a pale oil, provided it be pure ; but it is not always easy to obtain a light-coloured oil that is pure. Clove oil passes very readily from yellow to brown with age, so that in choosing a colourless sample you run great risk of obtaining an adulterated sample, for clove oil is one of the most adulterated substances in commerce.

Clove oil does not easily spread itself over the surface of a slide, but has a tendency to form very convex drops. This property makes it a very convenient medium for making minute dissections in. It also has the property of making tissues that have lain in it for some time very brittle. This brittleness is also sometimes very helpful in minute dissections.

These qualities may be counteracted if desired by mixing the clove oil with bergamot oil.

This is one of the most useful of clearers. According to BEHRENS (*Tabellen*, 3 ed., 1898, p. 33), it will clear from alcohol of 74 per cent.

It has a high index of refraction, and clears objects *more* than balsam mounting media. It dissolves celloidin (or collodion), and therefore should not be used for clearing sections cut in that medium, without special precautions.

New clove oil washes out basic tar colours more quickly than old.

128. Cinnamon (or Cassia) Oil greatly resembles clove oil, but is in general thinner, and is more highly refractive. An excellent medium, which I particularly recommend.

129. Oil of Bergamot (SCHIEFFERDECKER, *Arch. Anat. Phys.*, 1882 [Anat. Abth.], p. 206.—This oil clears 95 per cent. alcohol preparations and celloidin preparations quickly, and does not extract anilin colours. I think that this is a valuable medium.

Bergamot oil is, I believe, the least refractive of these essences, having a lower index than even oil of turpentine.

SUCHANNEK (*Zeit. wiss. Mik.*, vii, 1890, p. 158) says that bleached, colourless bergamot oil will not take up much water, whereas a green oil will take up as much as 10 per cent.

VAN DER STRICHT (*Arch. de Biol.*, xii, 1892, p. 741) says that bergamot oil will, with time, dissolve out the fatty granules of certain ova.

130. Oil of Origanum (NEELSEN and SCHIEFFERDECKER, *Arch. Anat. Phys.*, 1882, p. 204).—Ninety-five per cent. alcohol preparations are cleared quickly, and so are celloidin sections, without solution of the celloidin. Anilin colours are somewhat extracted.

For work with celloidin sections care should be taken to obtain *Ol. Origani Cretici* (" Spanisches Hopfenöl "), not *Ol. Orig. Gallici* (v. GIESON ; see *Zeit. wiss. Mik.*, iv, 1887, p. 482). Specimens of origanum oil vary greatly in their action on celloidin sections.

SQUIRE, in his *Methods and Formulæ*, etc., p. 81, says that origanum oil (meaning the commercial product) is nothing but oil of white thyme more or less adulterated (see next §), and that the product sold as Ol. Origani Cretici is probably oil of marjoram.

131. Oil of Thyme.—FISH (*Proc. Amer. Mic. Soc.*, 1893 ; *Zeit. wiss. Mik.*, xi, p. 503), following BUMPUS, says that for most of the purposes for which origanum oil has been recommended, oil of thyme will do just as well if not better. After one distillation of the crude oil of thyme it is of a reddish-brown colour, and is called the *red* oil of thyme; when again distilled it becomes colourless, and is distinguished as the *white* oil. The red oil is just as efficient as the white for clearing.

Schimmel and Co., in their Report of October, 1895, p. 69, state that in France white oil of thyme is adulterated with oil of turpentine to the extent of as much as 50 per cent.

132. Oil of Gaultheria.—Used by UNNA (*Monatschr. prakt. Derm., Ergänzungsh,* 1885, p. 53) for thinning balsam. The artificial oil, methyl salicylate, is recommended by GUÉGUEN (*Comp. Rend. Soc. Biol.*, v. 1898, p. 285) both as a dealcoholisation and clearing agent and as a solvent of paraffin. The refractive index is 1·53. It is, unfortunately, very sensitive to water.

133. Sandal-wood Oil (NEELSEN and SCHIEFFERDECKER, *loc. cit.*, § 131).—Very useful, but its high price is prohibitive.

134. Oil of Cajeput.—This oil is, I believe, frequently used as a clearer by the botanists. I have used it myself and found it to clear well, but to be rather thin. CARNOY and LEBRUN (*La Cellule*, xiii, 1897, p. 71) have found it useful for clearing celloidin sections. It dissolves celloidin very slowly and clears without shrinkage.

135. Oil of Turpentine.—Generally used for treating sections that have been cut in paraffin, as it has the property of dissolving out the paraffin and clearing the sections at the same time; but many other reagents, such as xylol, benzol, are preferable for this purpose. If used for alcohol objects, it causes considerable shrinkage, and *alters the structure of cells* more than any other clearing agent known to me. Turpentine has, I believe, the lowest index of refraction of all the usual clearing agents except bergamot oil; it clears objects *less* than balsam.

136. Carbolic Acid.—Best used in concentrated solution in alcohol. Clears instantaneously, even very watery preparations. This is a very good medium, but it is better avoided for preparations of soft parts which it is intended to mount in balsam, as they generally shrink by exosmosis when placed in the latter medium. It is, however, a good medium for celloidin sections.

137. GAGE'S **Mixture** (*Proc. Amer. Soc. Micr.*, 1890, p. 120).—Carbolic acid crystals melted, 40 c.c.; oil of turpentine, 60 c.c.

138. **Creosote.**—Much the same properties as carbolic acid. *Beech-wood* creosote is the sort that should be preferred for many purposes,—amongst others, for clearing celloidin sections, for which it is a very good medium.

139. **Anilin Oil.**—This is a rather important reagent on account of its ability to clear excessively watery objects. Common anilin oil will readily clear sections from 70 per cent. alcohol, and with certain precautions (for which see the paper of SUCHANNEK quoted below) objects may be cleared from watery media without the intervention of alcohol at all, This property renders anilin valuable in certain cases as a penetrating medium for preparing for paraffin imbedding. For ordinary work the usual commercial anilin will suffice; and it is immaterial whether it be colourless or have become brown through oxidation. For difficult work it is well to use a perfectly anhydrous oil. For directions for preparing this see SUCHANNEK, *Zeit. wiss. Mik.*, vii, 1890, p. 156, or the third edition of this work.

Anilin is chiefly used for clearing celloidin sections, and is sometimes found very valuable for this purpose.

140. **Xylol, Benzol, Toluol, Chloroform.**—Too volatile to be recommendable as clearing agents in which it is desired to *examine* specimens, but very useful for preparing paraffin sections for balsam. Of the three first-mentioned liquids, benzol is the most volatile, then toluol, and xylol is the least volatile, in the proportion of 4 : 5 : 9 (SQUIRE, *Methods and Formulæ*, p. 20). Chloroform is injurious to some delicate stains, but is in other respects an excellent de-alcoholisation agent, as it will take up a good deal of water, if any be left in the preparations. I consider it too volatile for use before balsam. Xylol is the best of these in that respect; but it has the defect of mixing very slowly with alcohol. I now generally bring my sections from alcohol first into chloroform, to remove the alcohol; then into xylol, and thence into the balsam.

Both xylol and toluol are liable to become acid if kept in only partially filled vessels.

141. Amyl Alcohol.—Janssens (*La Cellule*, xiv, 1898, p. 209) treats cover-glass preparations, taken from 95 per cent. alcohol, with amyl alcohol before mounting in damar or colophonium, with the view, if I understand rightly, of more efficaciously completing the dehydration of the preparations. I do not understand whether he mounts direct from the amyl alcohol or passes through an essence.

CHAPTER VII.

IMBEDDING METHODS—INTRODUCTION.

142. A word on Microtomes.—The freezing microtome is less than any other form adapted to the wants of the *zoologist*. The relations of the parts of the organs are deranged by the freezing and by the thawing, and the aqueous nature of the process prevents it from being readily applicable to the mounting of *series* of sections. The microtome of the zoologist should be an *imbedding* microtome.

Now there are two methods of imbedding in general use—the paraffin method and the celloidin method. It so happens that the most precise and beautiful microtomes that have been constructed are designed in view of the paraffin method, and cannot be applied, or at all events are much less adapted, to work with celloidin objects. If the anatomist cannot afford two instruments, he will perhaps do well not to choose one of those that are adapted *only* for paraffin, but to choose an all-round instrument, one that without being absolutely of the highest attainable precision in paraffin work will yet give sufficiently good results in that way, and will also cut in the wet way.

Amongst microtomes fulfilling these conditions I particularly recommend *the* THOMA *sliding microtome*, as made, in several sizes, by R Jung, Mechaniker in Heidelberg. For zoological and general histological work I recommend the *medium size* (No. 4), with the newest Naples object-holder and newest form of knife and knife-holder. See the description in CARPENTER'S *The Microscope*, p. 461, and the latest price list of R. Jung (which may be obtained from Mr. C. Baker, Optician, 244, High Holborn, London, W.C.).

The BECKER *microtome* is also very much to be recommended. It is made by Aug. Becker, Göttingen. Descriptions of two forms (Spengel and Schiefferdecker) will be found in *Journ. Roy. Mic. Soc.*, 1886, pp. 884 and 1084. The Naples object-holder can be fitted to the Becker microtome.

A very complete instrument of the sliding kind, made by REICHERT, is described by ALBRECHT in *Zeit. wiss. Mik.*, xvii, 1900, p. 159.

The above are *sliding microtomes*, that is instruments in which the object to be cut is a fixture during cutting, and the knife is moved on a slide and is only attached to its holder at one end. This arrangement will not allow the highest possible accuracy to be obtained with paraffin objects or any other hard objects. For with hard objects the knife in free to yield slightly on meeting the object, instead of cutting its way through it. This defect is fatal to the attainment of perfectly cut series of sections of equal thickness throughout. For the highest class of work it is necessary to employ a microtome in which *the knife is a fixture*, and fixed at both ends; the object being moved against it. The following instruments are constructed on this principle, and for accurate cutting of paraffin sections seem to me superior to any sliding microtome. They cannot be considered to be "all-round" instruments, because (although some of them are fitted with an arrangement for that purpose) they are not well adapted for giving to the knife the oblique position and slow motion requisite for cutting celloidin material or objects of very hard, or very soft, or very heterogeneous consistency. Also, the object is placed in an awkward position for orientation and observation whilst cutting.

The *Cambridge rocking microtome* (furnished by the Cambridge Scientific Instrument Company, Cambridge, price £4, or by Messrs. Swift and Son, or by Jung) is extremely simple and rapid, and cuts very level series of sections. It should be fitted with the adjustable object-holder, for precise orientation of the object. This microtome is also described in CARPENTER'S *The Microscope*, p. 469. Jung's form is more expensive than the English one, but contains several very useful improvements in details.

Rather more costly (£8 15*s*.) is the MINOT microtome made by E. Zimmermann, Mechaniker, 21, Emilien Strasse, Leipzig. A description and figures of this instrument will be found in *Zeit. wiss. Mik.*, ix, 1892, p. 176, or in *Journ. Roy. Mic. Soc.*, 1889, p. 143. This microtome cuts with very great rapidity. MAYER (*Grundzüge*) gives the preference to the form of this instrument made by A. Becker, of Göttingen, over that made by Zimmermann.

The most beautiful of all these instruments is the REINHOLD-GILTAY. It is made by J. W. GILTAY, Delft, and costs about £20. A description will be found in *Zeit. wiss. Mik.*, ix, 1893, p. 445, and in *Journ. Roy. Mic. Soc.*, 1893, p. 706; and a description of some later improvements in *Zeit. wiss. Mik.*, xv, 1898, p. 23, or *Journ. Roy. Mic. Soc.*, 1899, p. 106.

143. Imbedding Methods.—The processes known as Imbedding Methods are employed for a twofold end. Firstly, they enable us to surround an object, too small or too delicate to be firmly held by the fingers or by any instrument, with some plastic substance that will support it on all sides with firmness but without injurious pressure, so that by cutting sections through the composite body thus formed, the included object may be cut into sufficiently thin slices without distortion. Secondly, they enable us to fill out with the imbedding mass the natural cavities of the object, so that their lining membranes or other structures contained in them may be duly cut *in situ*; and, further, they enable us not only to surround with the supporting mass each individual organ or part of any organ that may be present in the interior of the object, but also to fill out or impregnate with it each separate cell or other anatomical element, thus giving to the tissues a consistency they could not otherwise possess, and ensuring that in the thin slices cut from the mass all the minutest details of structure will precisely retain their natural relations of position.

These ends are usually attained in one or two ways. Either the object to be imbedded is saturated by soaking with some material that is liquid while warm and solid when cold, which is the principle of the processes here called *Fusion Imbedding Methods;* or the object is saturated with some substance which whilst in solution is sufficiently fluid to penetrate the object to be imbedded, whilst at the same time, after the evaporation or removal by other means of its solvent, it acquires and imparts to the imbedded object sufficient firmness for the purpose of cutting. The collodion process sufficiently exemplifies this principle. If a piece of soft tissue be dehydrated, and soaked first in ether and then in collodion, and if the ether contained in the collodion be allowed slowly to evaporate, the tissue and mass of collodion which penetrates and surrounds it will acquire a consistency such as to admit of thin sections being cut from them. The methods founded on this principle are here called *Evaporation Imbedding Methods.*

In any of these processes the material used for imbedding is technically termed an "imbedding mass."

Amongst the very various methods of imbedding that have been proposed two are pre-eminently important—the paraffin method and the celloidin or collodion method ; indeed these are the only ones that have survived in general use.

The subject of the respective merits of paraffin and celloidin still affords matter for discussion. The case, however, seems to be a very simple one. Celloidin does not afford by a long way the thinnest sections that are obtainable with small objects. For such objects it is, therefore, not equal to the demands made by modern minute anatomy, and paraffin must be taken. On the other hand, paraffin (as at present employed) will only cut *very thin* sections with fairly small objects ; with objects of much over half an inch in diameter you cannot get with paraffin thinner sections than you can with celloidin ; and if you try to cut in paraffin objects of still greater size, say an inch and upwards, it will frequently happen that you will not get perfect sections at all, blocks of paraffin of this size having a tendency to split under the impact of the knife ; so that for very large objects celloidin generally gives better results, in this respect, besides presenting certain advantages for the manipulation and staining of the sections.

This defect is, however, much reduced by the employment of a softer paraffin than is usual. In this way STRASSER (*Zeit. wiss. Mik.*, ix, 1892, p. 7) has obtained series of frontal sections 30 μ thick through the entire human brain, in paraffin blocks measuring 10 × 15 cm.

I have not been able to satisfy myself that the preservation of the tissues is better in celloidin sections than in paraffin sections ; so that—convenience apart—the case remains as above stated,—paraffin for small sections, celloidin for large ones.

To these may be added aqueous masses, such as gum or gelatin, for very special cases. They may render great service in cases in which it is desired to *avoid dehydrating* the objects.

144. Imbedding Manipulations.—Imbedding in a melted mass, such as paraffin, is performed in one of the following ways. A little tray or box or thimble is made out of paper, some melted mass is poured into it ; at the moment when the mass has cooled so far as to have a consistency that will not allow the object to sink to the bottom, the object is placed on its surface, and more melted mass poured on until the object is covered by it. Or the paper tray being placed on cork, the object may be fixed in position in it whilst empty by means of pins and the tray filled with melted mass at one pour. The pins are removed when the mass is cold.

In either case, when the mass is cold the paper is removed from it before cutting.

To make **paper trays** proceed as follows. Take a piece of stout paper or thin cardboard, of the shape of the annexed figure (Fig. 1) ; thin (foreign) post-cards do very well indeed. Fold it along the lines *a a′* and *b b′*, then along *c c′* and *d d′*, taking care to fold always the same way. Then make the folds *A A′*, *B B′*, *C C′*, *D D′*, still folding the same way. To do this you apply *A c* against *A a*, and pinch out the line *A A′*, and so on for the remaining angles. This done, you have an imperfect tray with dogs' ears at the angles. To finish it, turn the dogs' ears round against the ends of the box, turn down outside the projecting flaps that remain, and pinch them down. A well-made post-card tray will last through several imbeddings, and will generally work better after having been used than when new.

Another method of folding the paper (Mayer) is described in the *Grundzüge*, p. 81.

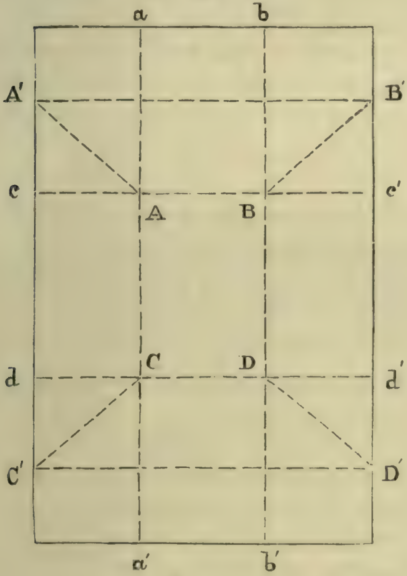

Fig. 1.

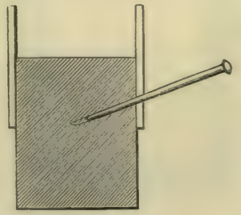

Fig. 2.

Giesbrecht now makes trays of photographic films, which being transparent facilitate orientation under the dissecting microscope.

To make **paper thimbles**, take a good cork, twist a strip of paper several times round it so as to make a projecting collar, and stick a pin through the bottom of the paper into the cork. For work with fluid masses, such as celloidin, the cork may be loaded at the bottom by means of a nail or piece of lead, to prevent it from floating when the whole is thrown into spirit or other liquor for hardening (Fig. 2).

Leuckhart's **Imbedding Boxes** are made of two pieces of type-metal (Fig. 3). Each of these pieces has the form of a carpenter's "square" with the end of the shorter arm triangularly enlarged outwards. The box is constructed by placing the two pieces together on a plate of glass which has been wetted with glycerin and gently warmed. The area of the box will evidently vary according to the position given to the pieces, but the height can be varied only by using different sets of pieces. Two sets will be sufficient for most work; one set of one centimetre in height, and one of two centimetres, each being eight centimetres in length, and three in breadth. To make the box paraffin-tight, so that it will hold the melted paraffin long enough in the liquid state to permit of the objects being carefully orientated in it, MAYER (*Mitth. Zool. Stat. Neapel*, iv, 1883, p. 429) first smears the glass plate with glycerin, then arranges the metal "squares," and then fills the box with collodion, which is poured out again immediately. As the ether evaporates, a thin layer of collodion remains behind, which suffices to keep the paraffin from running out. Even without the collodion, the mere cooling of the paraffin by the metal will generally suffice to keep it in long enough for orientation, if it is not in a superheated state when it is poured in.

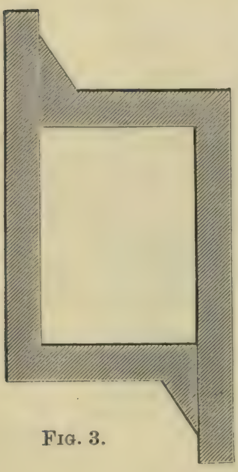

FIG. 3.

In such a collodionised box the paraffin may be kept in a liquid state by warming now and then over a spirit lamp, and small objects be placed in any desired position under the microscope (*Journ. Roy. Mic. Soc.* [N.S.], ii, p. 880).

A lighter form of "squares," made of brass, and devised by ANDRES, GIESBRECHT, and MAYER, is described *loc. cit.* (see *Journ. Roy. Mic. Soc.*, 1883, p. 913).

FRANKL (*Zeit. wiss. Mik.*, xiii, 1897, p. 438) builds up boxes with rectangular blocks of glass, which may be found convenient, but are more expensive than the metal squares.

SELENKA has described and figured another sort of apparatus having the same object. It consists of a glass tube, through which a stream of warm water may be passed and changed for cold as desired, the object being placed in a depression in the middle of the tube (see *Zool. Anz.*, 1885, p. 419). A simple modification of this apparatus, which anyone may make for himself, is described by ANDREWS in *Amer. Natural.*, 1887, p. 101; and a more complicated imbedding and orienting box, seldom necessary, is described by JORDAN, in *Zeit. wiss. Mik.*, xvi, 1899, p. 32.

For **small paraffin objects** the following procedure is very useful. The object is removed from the melted paraffin, the superfluous paraffin is removed by means of blotting-paper, and the object placed on a cylinder of paraffin. A piece of stout iron wire is now heated in the flame of a spirit lamp, and with it a hole is melted in the end of the cylinder; the specimen is pushed into the melted paraffin, and placed in any desired position. The advantages of the method lie in the quickness and certainty with which it can be performed.

There remains **the watch glass method.** Melt paraffin in a watch glass, and throw the object, previously well de-alcoholised and penetrated with a solvent, into it; or place the object in the watch glass, add solid paraffin, and heat. After the mass has hardened, cut out a block containing the object (*this is of course applicable to other masses,* such as celloidin). This should be done with a thin-bladed knife, *slightly* warmed. If paraffin be used you may, instead of cutting out a block, turn out the whole mass of paraffin by simply warming rapidly the bottom of the glass, but I find it is far safer to cut out a block. To facilitate the removal of the mass some persons lubricate the watch glass before pouring in the mass. To do this a drop of glycerin or, according to some, clove oil, should be smeared over it and wiped off with a cloth until hardly a trace of it remains. But this is not necessary.

As regards *small objects* at all events, I consider the watch glass process to be *the very best of any.*

For imbedding *very small* objects in this way certain precautions may be necessary in order not to lose them. SAMTER (*Zeit. wiss. Mik.*, xi, 1894, p. 469) saturates small unstained objects with paraffin that has previously been strongly coloured with alkanna extract, and then imbeds them in pure paraffin. The objects do not stain with the alkanna. RHUMBLER (*ibid.*, xii, 1895, p. 312 and xiii, 1896, p. 303) stains previously the objects themselves lightly with eosin dissolved in strong alcohol, and removes the stain from the sections with weak alcohol. See also *ibid.*, xiii, p. 200, a paper by Schydlowski; and in *Zeit. wiss. Zool.*, lviii, 1897, p. 144, a process of Borgert.

A watch glass provided at the bottom with a groove or trough, in which small objects may be made to collect, is described by Lefevre, *Journ. App. Mic.*, v, 1902, p. 280 (see *Journ. Roy. Mic. Soc.*, 1903, p. 233), and should be useful.

CHAPTER VIII.

IMBEDDING METHODS—PARAFFIN AND OTHER FUSION MASSES.

145. Saturation with a Solvent.—The first stage of the paraffin method consists in the saturation of the object with some substance which is a solvent of paraffin. The process is sometimes called a clearing process, since many of the substances used for infiltration are also "clearing" agents.

The process of saturation should be carefully performed with well-dehydrated objects in the manner described in § 123.

Saturation liquids being liquids that are, on the one hand, miscible with alcohol, and on the other hand good solvents of paraffin, are not quite as numerous as could be wished. Amongst them may be mentioned essence of turpentine, clove oil, bergamot oil, benzol, xylol, toluol, naphtha, oil of cedar-wood, chloroform, and anilin oil. But they are by no means all equally good, for few of them are as good solvents of paraffin as is desirable.

Turpentine penetrates well, and mixes readily with paraffin. I do not, however, recommend it, because in my experience it is of all others the clearing agent that is the most hurtful to delicate structures.

Clove oil penetrates well, and preserves delicate structures well; but it mixes very imperfectly with paraffin, and quickly renders tissues brittle.

Oil of bergamot mixes still more imperfectly with paraffin.

Benzol has been recommended by BRASS (*Zeit. wiss. Mik.*, ii, 1885, p. 301), and is now much used.

Toluol (or toluen) has been recommended by HOLL (*Zool. Anz.*, 1885, p. 223).

Xylol is said by M. HEIDENHAIN (*Kern und Protoplasma*, p. 114) to be a cause of shrinkage in cells. So it is, if you use it to de-alcoholise the specimens, as it mixes very badly with alcohol. But used *after oil of cedar*, or the like, it is very good, as it is one of the best of solvents of paraffin.

Naphtha has been recommended by WEBSTER (*Journ. Anat. and Physiol.*, xxv, 1891, p. 278). Dr. Webster writes me that a quality known as "Persian naphtha" is best for fine work, but the common pure naphtha is sufficient for ordinary work.

FIELD and MARTIN (*Zeit. wiss. Mik.*, xi, 1894, p. 10) recommend a light petroleum known as "petroleum-æther."

Sulphide of carbon has been lately recommended by HEIDENHAIN (*Zeit. wiss. Mik.*, xviii, 1901, p. 166) as being very penetrating, not being oxidising (which may be important in view of the preservation of some stains), and as being a very powerful solvent of paraffin. I think it will be found to be much too disagreeable and dangerous a reagent for ordinary work, and that it is *not necessary* even for delicate work.

Carbon tetrachloride and ligroïn have been recommended by PLEČNIK (*op. cit.*, xix, 1903, p. 328) and PRANTER (*ibid.*, p. 329) on the ground of not dissolving-out ossium-blackened fats.

For Guéguen's methyl salicylate, see § 132.

Chloroform mixes well with paraffin, and after evaporation in a paraffin bath (in the manner described in the next paragraph) leaves behind a pure and very homogeneous paraffin, having but little tendency to crystallise. But it is deficient in penetrating power, so that it requires an excessive length of time for clearing objects of any size; and it must be very thoroughly got rid of by evaporation in the paraffin bath, or by successive baths of paraffin, as if the least trace of it remains in the paraffin used for cutting it will make it soft. The process of removal requires a very long time, in some cases days. It ought therefore to be reserved for small and easily penetrable objects. Under suitable conditions, and properly employed, it is certainly one of the best, if not the very best of these media.

Cedar-wood oil is, according to my continued experience, for the reasons stated by me in *Zool. Anz.*, 1885, p. 563, for general work the *very best* clearing agent for paraffin imbedding. It penetrates rapidly, preserves delicate structure better than any clearing agent known to me, does not make tissues brittle, even though they may be kept for weeks or months in it, and has the great advantage that if it be not entirely removed from the tissues in the paraffin bath it will not seriously impair the cutting consistency of the mass; indeed, I fancy it sometimes improves it by rendering it less brittle. I do not mean to assert that it is in *all cases* the best, for for some fine work I think chloroform may give more accurate results. And it may often be indicated to employ the two reagents successively, as recommended by Apáthy, *see* next §.

In some cases anilin oil is indicated (see § 139).

146. The Paraffin Bath.—The objects having been duly saturated with a solvent, the next step is to substitute melted paraffin for the saturating medium.

Some authors lay great stress on the necessity of making the passage from the saturating agent to the paraffin as gradual as possible, by means of successive baths of mixtures of solvent and paraffin kept melted at a low temperature, say 35° C. With oil of cedar or toluol, at all events, this is not necessary. All that is necessary is to bring the objects into melted paraffin kept just at its melting point, and keep them there till they are thoroughly saturated ; the paraffin being changed once or twice for fresh only if the objects are sufficiently voluminous to have brought over with them a notable quantity of clearing agent.

If chloroform or other volatile agent be taken, choice may be made of two methods : either, as in Giesbrecht's method, the chloroform containing the object is heated to the melting-point of the paraffin, and the paraffin gradually added ; or, as in Bütschli's method, the objects are simply passed direct from chloroform into a solution of paraffin in chloroform, in which they remain until thoroughly impregnated (half to one hour), and which is then evaporated at the melting-point of the paraffin. Bütschli recommends a paraffin solution melting at 35°. (Such a solution is made of about equal parts of chloroform and paraffin of 50° melting-point.) Or, in the case of larger objects, instead of evaporating the chloroform (which is often a very long process, as the chloroform must be *completely* driven off, or the mass will remain too soft for cutting), Bütschli simply transfers them from the bath of paraffin solution to a bath of pure paraffin.

Giesbrecht's method (*Zool. Anz.*, 1881, p. 484), more fully stated, is as follows :

Objects to be imbedded are saturated with absolute alcohol and then brought into chloroform (to which a little sulphuric ether has been added if necessary, in order to prevent the objects from floating). As soon as the objects are saturated with the chloroform, the chloroform and objects are gradually warmed up to the melting-point of the paraffin employed, and during the warming small pieces of paraffin are by degrees added to the chloroform. So soon as it is seen that no more bubbles are given off from the objects, the addition of paraffin may cease, for that is a sign that the paraffin has entirely displaced the chloroform in the objects. This displacement having been a *gradual* one, the risk of shrinkage of the tissues is reduced to a minimum.

Mayer (*Grundzüge*, p. 88) first saturates the objects with benzol, which should be changed once or twice so as to make sure of removing all the alcohol, and then adds to the pure benzol some small pieces of paraffin, and lets them dissolve in the cold. After several hours (up to eighteen) the whole is brought in an open vessel on to the cold water-bath, the bath is then warmed gradually so as to attain a temperature of 60° C. in about two hours, and as fast as the benzol evaporates melted paraffin is added to it. Lastly, the paraffin is changed once before the definitive imbedding. He rarely leaves objects over night in the water-bath.

Apáthy (*Mikrotechnik*, pp. 149, 150) first clears with oil of cedar, then brings the objects (by the process described §123) into a solution of paraffin in chloroform saturated at the temperature of the laboratory. The objects remain in the chloroform-paraffin solution for from one to three hours, without warming, until all the cedar oil is soaked out of them. The whole is then warmed on the water-bath or oven to a few degrees above the melting-point of the paraffin intended to be used for imbedding, and the object is brought into a mixture of equal parts of paraffin and chloroform, being suspended therein near the top on a bridge made of hardened filter paper (or in a special apparatus to the same end, not yet described). It remains in this mixture, at the temperature of the oven, for one to three hours, and lastly is brought (still on the paper bridge or in the apparatus) into pure paraffin, where it remains for half an hour to two hours.

Heidenhain (*Festschr. f. Koelliker*, Leipzig, 1892, p. 114) used to clear with bergamot oil, and pass into pure paraffin through mixtures of the oil and paraffin. He now prefers sulphide of carbon, *see last* §. Rabl also (*Zeit. wiss. Mik.*, xi, 1894, p. 164) employs bergamot oil.

The practice of giving successive baths first of soft and then of hard paraffin, which has been frequently advised, appears to me *entirely illusory.*

It is important to keep the paraffin *dry*—that is protected from vapour of water during the bath.

It is still more important to keep it *as nearly as possible at melting-point.* If it be heated for some time to a point much over its normal melting-point, *the melting-point will rise,* and you will end by having a harder paraffin than you set out with. And as regards the preservation of tissues, of course the less they are heated the better. Overheating, as well as prolonged heating, tends, amongst other things, to make tissues brittle.

The *duration of the bath* must, of course, vary according to the size and nature of the object. An embryo of 2 to 3 millimetres in thickness ought to be thoroughly saturated after an hour's bath, or often less, if cedar oil has been used for clearing. Many workers habitually give much longer baths, I think often longer than necessary. I take as a guide, generally, the length of time the object has taken to clear in the cedar oil, assuming that the *warm* melted paraffin ought to penetrate at least as quickly as the *cold* oil; and then allowing somewhat longer, say as much again. in order to be on the right side.

In any case the preparations should be *cooled* (see below, §150) *as soon as saturated.* If left for very many hours in a warm bath, as is sometimes done, delicate structures may be seriously injured. It is therefore important both to employ a paraffin of the lowest melting-point that will give good sections (see below, § 159), and to abbreviate the warm bath as much as possible.

147. Water-baths and Ovens.—It is important that the paraffin should not be exposed to a moist atmosphere whilst it is in the liquid state. If a water-bath be used for keeping it at the required temperature provision should be made for protecting the paraffin from the steam of the heated water.

A very convenient apparatus for this purpose is that of Paul Mayer, or "Naples water-bath," which will be found described at p. 146 of *Journ. Roy. Mic. Soc.*, 1883. It may be procured from the opticians, *e. g.* Mr. Baker. See also *Amer. Natural.*, 1886, p. 910, and *Journ. Roy. Mic. Soc.*, 1887, p. 167.

Amongst apparatus arranged for heating by means of petroleum or similar combustibles other than gas may be mentioned the stove manufactured and sold by F. SANTORIUS, Göttingen (*Zeit. wiss. Mik.*, x, 1893, p. 161), and that of ALTMANN (*ibid.*, p. 221, cf. *Centralb. f. Bakteriol.*, xii, 1892, p. 654); also that of KARAWAIEW (*Zeit. wiss. Mik.*, xiii, 1896, p. 172).

For the elaborate electrically-heated stove of REGAUD (price £10), see *Zeit. wiss. Mik.*, xx, 1903, p. 138, or the advertisement in *Anat. Anz.*, xxiv, No. 7; for that of MARK, *Amer. Natural*, xxxvii, 1903, No. 434.

For ordinary work it is by no means necessary, though it may be convenient, to possess one of these costly and complicated heat-regulating contrivances; in default of gas, a spirit lamp with the wick well turned down, or a night-light, will suffice to keep the temperature constant enough, if watched occasionally.

148. Imbedding IN VACUO.—There are objects which, on account of their consistency or their size, cannot be penetrated by paraffin in the ordinary way, even after hours or days in the bath. For such objects the method of imbedding under a vacuum (strictly, under diminished atmospheric pressure), renders the greatest service. It not only ensures complete penetration in a very short time—a few minutes—but it has the further advantage of *preventing any falling in of the tissues*, such as may easily happen with objects possessing internal cavities if it be attempted to imbed them in the ordinary way.

The principle of this method is that the objects are put through the paraffin bath *in vacuo*. In practice this may be realised by means of any arrangement that will allow of maintaining paraffin at the necessary temperature for keeping it fluid under a vacuum.

The apparatus of HOFFMANN is described and figured at p. 230 of *Zool. Anz.*, 1884. In this arrangement the vacuum is produced by means of a pneumatic water aspiration pump, the vessel containing the paraffin being placed in a desiccator heated by a water-bath and furnished with a tube that brings it into communication with the suction apparatus. This arrangement is very efficacious and very simple if the laboratory possesses a supply of water under sufficient pressure.

In order to obtain the requisite vacuum without the aid of water under pressure, a simple little apparatus has been designed by FRANCOTTE (*Bull. Soc. Belg. Mic.*, 1884, p. 45). In this the vacuum is produced by the condensation of steam.

FOL (*Lehrb.*, p. 121) employs the vacuum apparatus of Hoffmann, but simplifies the arrangement for containing the paraffin. The paraffin is contained in a stout test-tube furnished with a rubber stopper traversed by a tube that puts it into communication with the pump. The lower end of the test-tube dips into a water-bath. You pump out the air once or twice, wait a few minutes to make sure that no more bubbles rise, then let the air in, turn out the object with the paraffin (which by this time will have become abnormally hard), and re-imbed in fresh paraffin.

See also a paper by PRINGLE, in *Journ. Path. and Bacteriol.*, 1892, p. 117; or *Journ. Roy. Mic. Soc.*, 1892, p. 893; and one by KOLSTER, in *Zeit. wiss. Mik.*, xviii, 1901, p. 170.

149. Imbedding and Orientation.—As soon as the objects are thoroughly saturated with paraffin they should be imbedded by one of the methods given above (§ 144). *If the watch glass method be followed* the paraffin bath will naturally have been given in the watch glass used for imbedding, and *no special imbedding manipulation may be necessary*; it may suffice to cool the paraffin, cut out a block containing the object, and mount it on the microtome.

But it may be desirable to have the object fixed in the cooled block in a precisely-arranged position, and, above all, in a more precisely-marked position.

Very small objects may be taken out of the paraffin with a needle or small spatula, and put to cool on a block of glass, then imbedded in position for cutting on a cone of paraffin by means of a heated needle in the manner described above (§ 144). In the use of the needle it should be noted that it is important *to melt as little paraffin as possible at one time*, in order that that which is melted may cool again as rapidly as possible.

For BOVERI's plan for imbedding numerous very small objects see § 599, and for that of LAUTERBORN see "Protozoa."

If a more precise orientation be required, one of the following methods may be adopted:

The method of PATTEN (*Zeit. wiss. Mik.*, xi, 1894, p. 13) is especially useful when one desires to orient accurately large numbers of small and similar objects. You get some writing paper of the sort that is made with two sets of raised parallel lines running at right angles to each other ("linen cloth paper"). Small strips are cut from this, and at suitable intervals along them small drops of a mixture of collodion and clove oil, of about the consistency of thick honey, are arranged close together along one of the ribs that run lengthwise. The objects to be imbedded are cleared in clove oil or oil of bergamot—not turpentine. They are taken one by one on the point of a knife, and after the excess of oil has been drawn off, are transferred each to a drop of the collodion mixture. They may be adjusted therein under the dissecting microscope, and will stay in any required position. When half a dozen or more objects have been oriented in reference to the cross lines (which are to be parallel to the section planes) the whole thing is placed in turpentine. This washes out the clove oil and fixes the objects very firmly to the paper. The paper with the attached objects is now passed through the bath of paraffin and imbedded in the usual way. After cooling on water the block is trimmed and the paper peeled off, leaving the objects in the paraffin close to the under surface of the block. This surface is now seen to be marked by the orienting lines of the ribbed paper, and also by any record numbers which may before imbedding have been written with a soft pencil on the paper.

A somewhat more complicated form of this process has been described by Woodworth, *Bull. Mus. Comp. Zool.*, xxxviii, vol. xxv, 1893, p. 45.

A similar process has also been described by Field and Martin in *Zeit. wiss. Mik.*, xi, 1894, p. 11, small strips of gelatin being used instead of paper.

Hoffmann (*ibid.*, xv, 1899, p. 312, and xvii, 1901, p. 443) prefers to take, instead of the ribbed paper, glass slips ruled with a diamond, and to completely imbed the objects in large drops of clove oil collodion (equal parts), allowed to stand for twenty-four hours in an open vessel). The drops are caused to set in xylol instead of turpentine. See also Samter, *ibid.*, xiii, 1897, p. 441; Jordan, *ibid.*, xvi, 1899, p. 33; and Peter, *Verh. Anat. Ges.*, xiii Vers., 1899, p. 134.

Denne (*Journ. Appl. Mic.*, iii, 1902, p. 888) imbeds on disks of paper held at the bottom of glass tubes containing the paraffin by bent wires, by means of which a cylinder of paraffin containing the object may be lifted out as soon as cool.

Wilson (*Zeit. wiss. Mik.*, xvii, 1900, p. 169) makes orientation lines by imbedding alongside the objects strands of osmium-blackened nerve-fibres.

See also the article "Plastische Rekonstruction" in the *Encycl. mîk. Technik.*

150. Cooling the Mass.—Whatever method of imbedding and orientation in the molten paraffin has been employed, the important point now to be attended to is that *the paraffin be cooled as rapidly as possible.* The object of this is to prevent crystallisation of the paraffin (which may happen if it be allowed to cool slowly) and to get as homogeneous a mass as possible.

If the watch glass method be adopted, float the watch glass with the paraffin and objects on to cold water. Do not let it sink till all the paraffin has solidified. When cool, cut out blocks containing the objects; do this with a *slightly* warmed scalpel.

If paper trays be taken, cool them on water, holding them above the surface with only the bottom immersed until all the paraffin has solidified, as if you let them go to the bottom at once you will probably get cavities filled with water formed in your paraffin. Or you may put them to cool on a block of cold metal or stone.

Preparations imbedded in the metal "squares" are cooled in a similar manner.

Selenka cools the mass by passing a stream of cool water through the imbedding tube described above (§ 144). Mayer cools the mass in the paraffin-tight moulds (§ 144) by passing cold water through a special movable water-bath, which allows of the arrangement of the objects by transmitted light under a dissecting microscope, see *Mitth. Zool. Stat. Neapel*, iv, 1883, p. 429; *Intern. Monatsschr. Anat. Hist.*, iv, 1887, p. 39. A complicated apparatus for the same purpose is described by Meissner (*Zeit. wiss. Mik.*, xviii, 1902, p. 286).

The objects having been mounted on the carrier of the microtome in position for cutting, pare the blocks to the proper shape (next §), and sufficiently close down to the objects, and go round them with a lens. If any bubbles or cavities or opaque spots be present, prick with a heated needle till all is smooth and homogeneous. The same should be done if any cavities present themselves in the course of cutting. In bad cases, re-imbed.

151. Shape and Orientation of the Block of Mass to be cut.—Solid paraffin varies enormously in hardness according to the temperature of its surroundings. It must therefore be taken of a melting-point *suitable to the temperature of the laboratory*, harder (*i. e.* of higher melting-point) in summer, softer in winter. On this point see § 159.

As to the shape and orientation to be given to the block to be cut, these differ accordingly as the cutting is done with a slanting knife or a square-set knife (see next §). In the first case, the block is best trimmed to a three-sided prism, and orientated as in Fig. 4, so that the knife enters it at the angle *a* and leaves it at the angle *c*. When the section is cut it will adhere to the knife only by the angle *c*, and can thus most readily be removed by means of a brush or needle. The object itself should come to lie in the block close to the line *b c*, so that the knife at first cuts only paraffin, and that if the section begins to *roll* it may be caught and held down by a brush or section-stretcher before the object itself is reached. For the square-set knife the block is best trimmed to a four-sided prism, and orientated as in the first case, so that the knife first touches one angle, if only *isolated* sections are to be cut. But if *ribbons* (see § 155) are to be cut, the block must be orientated with one of its sides parallel to the

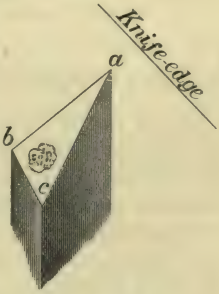

Fig. 4.

knife-edge, and the opposite side must be strictly parallel to this one.

For NOACK's simple apparatus for accurately orientating small blocks, see *Zeit. wiss. Mik.* xv, 1899, p. 438, or *Journ. Roy. Mic. Soc.*, 132, 1899, p. 550.

For ETERNOD'S machine for trimming blocks to true cubes, see *Zeit. wiss. Mik.*, xv, p. 421, and for that of SCHAFFER, *ibid.*, xvi, 1900, p. 417. I think these devices decidedly useful for accurate work.

152. Knife Position.—The position to be given to the knife may be considered under two heads, viz. its *slant* and its *tilt*.

By the *slant* of the knife is meant the angle that its edge makes with the line of section: that is, with the line along which it is drawn through the object (or along which the object moves across it in the case of microtomes with fixed knives). The position is *transverse* when the edge makes an angle of 90° with the line of section, or the knife in that case is said to be set *square*. It is *oblique* or *slanting* when it makes a smaller angle with that line.

It is a mistake to suppose that these two positions differ in that in the transverse position the knife acts as a wedge or chisel, forcing its way straight through the object, whilst in the oblique position it acts as a saw, its edge being *drawn along* through the object, as can be done in free-hand cutting. On the contrary, in both cases the knife acts merely as a wedge, and no microtome in general use at the present time * affords a drawing movement such as can be given by the hand. In either position of the knife *no point of the object is ever touched by more than one point of the cutting-edge.* The difference between the effect of the two positions is merely that the oblique position affords *a more acute-angled wedge* than the transverse one.

It does so for the following reasons:—Neglecting for the moment the distinction between the cutting-facets and the surfaces of the blade (which are distinct usually because they are not ground to the same angle),† it is clear that the knife itself is a wedge, the angle of which depends on the relation between the height of its base and the distance from the base to the edge. With the same base the angle becomes more acute the greater the distance from edge to base. Now by slanting the knife we can effect what is equivalent to an increase in the distance from edge to base; for we can thus increase the distance between the point of the edge which first touches the object, and the point of the back (strictly, of the back edge of the under cutting-facet) which last leaves it. When the knife is set transversely, the line along which any point of it traverses the object is the shortest possible from edge to base of the wedge, and the *effective* angle of wedge is the least acute obtainable with that knife. But if it is set as obliquely as possible, the line along which any point of it traverses the object traverses the knife from heel to toe, that is, along the greatest possible distance from edge to base, and therefore affords *practically* a much more acute-angled wedge than in the first case; and so on, of course, for intermediate positions. (See the stereometrical constructions of these relations by SCHIEFFERDECKER, *op. cit.*, p. 115; and also, with more instructive figures, APÁTHY, "Ueber die Bedeutung des Messerhalters in der Mikrotomie," in *Sitzber. med.-naturw. Section d. Siebenbürgischen Museumvereins*, Bd. xix, Heft. 7, p. 1 (Kolozsvár, 1897, A. K. Ajtai).

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* A microtome with drawing motion to the knife is described by BECK in *Zeit. wiss. Mik.*, xiv, 1897, p. 324; also one by THATE in *Zeit. angew. Mik.*, 1900, p. 73 (*Journ. Roy. Mic. Soc.*, 1900, p. 645).

† The edge of a microtome knife is composed of two plane surfaces—the upper and lower cutting-facets, which meet one another at an acute angle, the cutting-edge, and posteriorly join on to the upper and lower surfaces of the blade (see some good figures of differently shaped knives in BEHRENS, KOSSEL und SCHIEFFERDECKER, *Das Mikroskop.*, p. 115, *et seq.*; and in APÁTHY'S paper quoted below). It will be seen that the two facets together form a wedge welded on to the blade by the base.

Very large objects are best cut with the slanting knife, and so are all objects of very heterogeneous consistency, such as tissues that contain much chitin or much muscular tissue. In general all very difficult objects are better cut with the slanting knife than the transverse one (and better with a slowly-working sliding microtome than with a quick-working Rocker or the like). Soft masses, such as gelatin or celloidin cut wet, can only be cut with the slanting knife. The slanting position causes less *compression* of sections than the transverse one. It has the defect of producing rolling in paraffin sections more easily than the transverse position. The latter is the proper position for cutting ribbons of sections from paraffin.

By the *tilt* of the knife is meant the angle that a plane passing through its back and edge makes with the plane of section: or, practically, the greater or less degree of elevation of the back above the edge (it is not to be confounded with the inclination of the long axis of the knife to the horizon; any accidental inclination that this may have is a matter of no moment).

The question of the proper tilt to be given to the knife under different circumstances has been investigated by APÁTHY, *loc. cit. supra.* He concludes—(1) The knife should always be tilted somewhat more than enough to bring the back of the under cutting-facet clear of the object. (2) It should in general be less tilted for hard and brittle objects than for soft ones; therefore, *cæteris paribus*, less for paraffin than for celloidin. (3) The extent of useful tilt varies between 0° and 16° or occasionally 20°. (4) Excessive tilt causes rifts (longitudinal) in the paraffin, also furrows that in bad cases split up the section into narrow ribbons. It also makes sections roll. Also it may cause the knife not to bite, thus causing sections to be missed. Or it may give an undulatory surface to the sections, owing to vibrations set up in the knife, which may be heard as a deep humming tone. Further, I would add, excessive tilt may cause the knife to act as a scraper, carrying away portions of tissue bodily from their places. Excessive tilt may often be recognised by the knife giving out a short metallic sound just as it leaves the object. For knives with plane under surfaces it is seldom advisable to give less than 10° tilt. Knives with concave under surfaces, on the contrary, may require to be placed almost horizontal. Jung's knife-holders give mostly a tilt of about 9°, which is only enough for cutting ribbons with hard paraffin.

A knife with too little tilt will often cut a second section, or fragments of one, without the object being raised, showing that during the first cut the object was pressed down by the knife, and recovered itself afterwards. This fault is denoted by the ringing tone given out by the knife on passing *back* over the object before the latter is raised. Such a knife gives out a dull rattling sound whilst cutting. Too little tilt causes folding or puckering of sections, and does not allow of the cutting of the thinnest possible sections, as the edge does not bite enough.

Ribbon section-cutting (§ 155) requires a relatively hard paraffin and less tilt. With celloidin it is very important to avoid insufficient tilt, as the elastic celloidin yields before an insufficiently tilted knife and is not cut.

The tilt of the knife is given to a certain extent by the knife-holder sold with the microtome. It is more accurately *regulated* by means of mechanical contrivances, of which the most simple are the horseshoe-shaped wedges of NEUMAYER (see Jung's price-list). A pair of these, each ground to the same angle, is taken, and one of them placed under, and the other over, the clamping-arm of the knife-holder. Three pairs, having different degrees of pitch, are supplied, and are sufficient for most work. Other contrivances to the same end consist of knife-holders that permit of rotating the knife on its long axis, and, though more costly, will be found a great convenience where much section-cutting has to be done. For these see Jung's price-list, and various recent papers in *Zeit. wiss. Mik.*, also that of APÁTHY, in the paper quoted above (very complicated), and especially the description of the two latest of Jung, viz. his model *l* and model *n*, by MAYER and SCHOEBEL, in *Zeit. wiss. Mik.*, xvi, 1899, p. 29 (see figure of model *l* in *Journ. Roy. Mic. Soc.*, 132, 1899, p. 546).

153. Cutting and Section-stretching.—Paraffin sections are cut *dry*,—that is, with a knife not moistened with alcohol or other liquid. By this means better sections are obtained, but a difficulty generally arises owing to the tendency of sections so cut to curl up on the blade of the knife. It is sometimes difficult by any means to unroll a thin section that has curled. To prevent sections from *rolling*, the following points should be attended to.

First and foremost, the paraffin *must not be too hard*, but must be taken of a melting-point *suitable to the temperature of the laboratory*, § 159.

If, after cutting has begun, the paraffin be found to be too hard, it may be softened by placing a lamp, or any convenient source of heat, near the imbedded object. But then, the paraffin being warmed most on the side nearest the lamp, becomes softer on that side, and the sections have a tendency to become compressed and puckered-in on that side.

If, on the contrary, the paraffin be found too soft, it may be hardened by exposing it to the cooling influence of a lump of ice.

It is often sufficient to moderate the temperature of the room by opening or closing the window, stirring the fire, setting up a screen, or the like.

Secondly, the knife should be set square, for the oblique position encourages rolling, and the more the knife is oblique the more do the sections roll. Not that a square-set knife will always cure rolling! But it diminishes the tendency to roll.

Thirdly, it is better to cut ribbons than disconnected sections; ribbons of sections will often cut perfectly flat, even when the same mass will only give rolled sections if cut disconnectedly. For if a section has only a slight tendency to curl, it will be held down flat by adhesion to the one preceding it.

Mechanical means may be employed. The simplest of these is as follows:

During the cutting the edge of the section that begins to curl is caught and held down on the blade of the knife by means of a small camel-hair brush with a flat point, or by a small spatula made by running a piece of paper on to the back of a scalpel. Or the section is held down by means of an instrument called a "section-stretcher." This consists essentially of a little metallic roller suspended over the object to be cut in such a way as to rest on its free surface with a pressure that can be delicately regulated so as to be sufficient to keep the section flat without in any way hindering the knife from gliding beneath it.

See the descriptions of various forms of section-stretchers, *Zool. Anzeig.*, vol. vi, 1883, p. 100 (SCHULTZE); *Mitth. Zool. Stat. Neapel*, iv, 1883, p. 429 (MAYER, ANDRES, and GIESBRECHT); *Arch. mik. Anat.*, xxiii, 1884, p. 537 (DECKER); *Bull. Soc. Belg. Mic.*, x, 1883, p. 55 (FRANCOTTE); *The Microscope*, February, 1884 (GAGE and SMITH); WHITMAN'S *Meth. in Mic. Anat.*, 1885, p. 91; *Zeit. wiss. Mik.*, iv, 1887, p. 218 (STRASSER); *ibid.*, x, 1893, p. 157 (BORN). The best are those of Mayer and Born. It must be allowed that all these instruments are difficult to use, and that if they are not perfectly adjusted they may easily injure the sections. And they are less necessary than formerly, now that good processes for flattening out sections have been worked out (see § 156).

Another plan is to allow the sections to roll, but to control the rolling. To this end, the block of paraffin is pared to the shape of a wedge five or six times as long as broad, the object being contained in the broad part, and the edge turned towards the knife (see Fig. 4). The sections are allowed to roll and come off as coils, the section of the object lying in the outermost coil, which will be found to be a very open one—indeed, very nearly flat. Lay the coil on a slide with this end downwards, warm gently, and the part containing the object will unroll completely and lie quite flat.

A defect opposite to that of the rolling of sections is the *compression* and the crumpling or puckering of sections, indicating that the paraffin has been compressed by the knife instead of being merely cut true by it. Such sections, besides showing creases or folds, have a smaller area than that of the block from which they are cut. This is a bad fault, for the compression may obliterate important cavities or efface important limits between cell-layers, etc. It may be caused by a badly-cutting knife, and is very easily caused by the paraffin being too soft. To prevent it, correct the knife or cool the paraffin, or re-imbed in harder paraffin. If the crumpling has not gone so far as to cause the folds of the sections to adhere to one another, the sections may be perfectly cured by flattening on water; see § 156.

Devices for heating or for cooling the knife, with a view to the improvement of cutting, have been described; see VAN WALSEM in *Zeit. wiss. Mik.*, xi, 2, 1894, p. 218; also Jung's price list. I have myself sometimes found it advantageous to warm the knife.

154. Cutting Brittle Objects (Collodionisation).—Some objects are by nature so brittle that, notwithstanding all precautions taken in imbedding and previous preparation, they break or crumble before the knife, or furnish sections so friable that it is impossible to mount them in the ordinary way without some impairment of their integrity. Ova are frequently in this case. A remedy for this state of things consists in covering the exposed surface of the object just before cutting each section with a thin layer of collodion, which serves to hold together the loose parts of even the most fragile sections in a wonderfully efficacious way; and the same treatment applied to tissues which are not specially fragile will enable the operator to *cut sections considerably thinner* than can be obtained in the usual way. BÜTSCHLI has obtained in this manner sections of less than 1 μ in thickness.

The primitive form of the process was to place a drop of collodion on the free surface of each section just before cutting it. But this practice has two defects; the quantity of collodion employed sensibly softens the paraffin, and the thick layer of collodion when dry causes the sections to roll.

MARK (*Amer. Natural.*, 1885, p. 628; cf. *Journ. Roy. Mic. Soc.*, 1885, p. 738) gives the following directions:

"Have ready a little very fluid collodion in a small bottle, through the cork of which passes a small camel-hair brush, which just dips into the collodion with its tip. The collodion should be of such a consistency that when applied in a thin layer to a surface of paraffin it dries in two or three seconds without leaving a shiny surface. Collodion of this consistency does not produce a membrane on the paraffin in drying, and therefore has no tendency to cause sections to roll. It has further the advantage that it penetrates to a certain depth below the surface of the preparation, and fixes the deeper layers of it in their places. The collodion must be diluted with ether as soon as it begins to show signs of leaving a shiny surface on the paraffin.

"Take the brush out of the collodion, wipe it against the neck of the bottle, so as to have it merely moist with collodion, and quickly pass it over the free surface of the preparation. Care must be taken not to let the collodion touch the vertical surfaces of the paraffin, especially not the one which is turned towards the operator, as that will probably cause the section to become stuck to the edge or under surface of the knife. As soon as the collodion is dry, which ought to be in two or three seconds, cut the section, withdraw the knife, and pass the collodion brush over the newly-exposed surface of the paraffin. Whilst this last layer of collodion is drying, take up the section from the knife and place it with the collodionised surface downwards on a slide prepared with fixative of Schaellibaum. Then cut the second section, and repeat the manipulations just described in the same order. A skilful operator can cut ribbons of sections, collodionising each section."

HENKING (*Zeit. wiss. Mik.*, iii, 1886, p. 478) objects to the above process that the ether of the collodion softens the paraffin, and proposes a solution of *paraffin* in absolute alcohol. The solution is made by scraping paraffin into absolute alcohol.

For extremely brittle objects, such as ova of Phalangida, the same author recommends a thin (light yellow) solution of *shellac* in absolute alcohol.

HEIDER (*Embryonalentw. v. Hydrophilus*, 1889, p. 12; cf. *Zeit. wiss. Mik.*, viii, 1892, p. 509) employs a solution made by mixing a solution of *gum mastic* in ether, of a syrupy consistency, with an equal volume of collodion, and diluting the mixture with ether until quite thin and liquid.

Rabl (*ibid.*, xi, 2, 1894, p. 170) employs *superheated paraffin*, kept at a temperature of about 100° C. on a water-bath. This plan has the advantage of efficiently filling up any cavities there may be in the objects, and also of preventing the sections from rolling. A complicated development of this process is described by Lendenfeld in *Zeit. wiss. Mik.*, xviii, 1901, p. 18.

Apáthy (*Mikrotechnik*, p. 183) employs a 1 per cent. solution of celloidin, allows the sections to roll, and unrolls them by the water-process (§ 156).

Jordan (*Zeit. wiss. Mik.*), adds 5 drops of oil of cedar to 15 c.c. of the solution of celloidin, and finds that rolling is prevented.

155. Ribbon Section-cutting.—If a series of paraffin sections be cut in succession and not removed from the knife one by one as cut, but allowed to lie undisturbed on the blade, it not unfrequently happens that they adhere to one another by the edges so as to form a chain or ribbon which may be taken up and transferred to a slide without breaking up, thus greatly lightening the labour of mounting a series. The following appear to me to be the factors necessary for the production of a ribbon.

First, the paraffin must be of a *melting-point* having a certain relation to the temperature of the laboratory. I find that *small* sections can always be made to chain when cut from a good paraffin of 50° C. *melting-point in a room in which the thermometer stands at* 16° *to* 17° C. But see on this point § 159. Secondly, the *knife should be set square.* Thirdly, the block of paraffin should be trimmed so as to present a straight edge parallel to the knife edge ; and the opposite edge should also be parallel to this. Fourthly, the sections ought to be *cut rapidly*, with swift strokes. For it is the sharp impact of the knife that causes the sections to cohere. It is by no means necessary for this purpose to have recourse to special mechanical contrivances, as in the so-called ribbon microtomes. The Thoma microtome well flooded with oil is sufficient. But the automatic microtomes, and amongst them the Cambridge Rocking Microtome, the Reinhold-Giltay, and the Minot, are certainly most advantageous for this purpose.

Various plans, such as coating the edges of the paraffin with softer paraffin, or with Canada balsam, or the employment of specially prepared paraffin, have been recommended, with the idea that they help the sections to stick. I find that none of these devices are necessary. For the prepared paraffin of Spee see below, § 160.

Mayer, however (*Grundzüge*, p. 98), remarks hereon that though coating with a softer paraffin is not necessary when soft paraffin is taken for imbedding, yet if a paraffin of 55° to 60° melting-point is used, it is absolutely necessary to coat it with softer, for sections of 10 μ thickness, and at least advisable for thinner ones. To coat the block, take paraffin of about 40° C. melting-point, melt it, heat it to about 80° on the water-bath, dip the block into it for an instant, and rapidly turn it over so that the fluid paraffin may run down away from the top part as much as possible. Allow it to cool, and pare away again the soft paraffin from the two sides that are not to be arranged parallel to the knife. Large blocks may have two coatings given them.

It sometimes, though rarely, happens that the ribbon becomes *electrified* during the cutting, and twists and curls about in the air in a most fantastic and undesirable manner. It may be got flat by warming slightly.

156. Section-flattening.—The sections having been obtained may be cleared and mounted at once if they are quite perfect, that is, neither rolled nor creased nor compressed. But should they *in the least degree* show any of these defects, they must first be unrolled or smoothed, or *expanded* to their proper dimensions. It is most important not to neglect this point, as is often done in the case of sections that are neither rolled nor crumpled, but are *compressed*, as shown by their being of smaller area than the block from which they have been cut.

The most efficacious plan is combined treatment with fluid and heat. The sections are either floated on to the surface of warm water or warm alcohol contained in a suitable dish, which causes them to flatten out perfectly, and are then transferred to a slide, by floating them into position, or otherwise. Or the slide has a layer of water spread over it, the sections are laid on the water, and the slide is heated (to somewhat below the melting-point of the paraffin) until the sections flatten out, which happens in a few seconds. The method can be made available for fixing *series of sections* to the slide; for which see § 200.

A special water-bath for flattening sections is described by Nowak in *Zeit. wiss. Mik.*, xii, 1896, p. 447).

Van Walsem (*ibid.*, xi, 1894, p. 228) describes a plan according to which the sections are arranged on a strip of parchment-paper which is moistened and passed over a warmed cylinder revolving in water on the principle of a postage-stamp dampener.

157. Clearing and Mounting.—The sections having been duly smoothed by one of these processes, and duly fixed to the slide (unless it is desired to keep them loose) all that now remains is to get rid of the paraffin and mount or stain as the case may be. Many solvents of paraffin have been recommended for freeing sections from the paraffin with which they are infiltrated :—Turpentine, warm turpentine, a mixture of 4 parts of essence of turpentine with 1 of creasote, creasote, a mixture of turpentine and oil of cloves, benzin, toluol, xylol, thin solution of Canada balsam in xylol (only applicable to very thin sections), hot absolute alcohol, naphtha, or any other paraffin oil of low boiling-point. Of these xylol and toluol are generally in most respects the best. Benzol and chloroform are too volatile for safe manipulation.

If the slide be warmed to the melting-point of the paraffin, a few seconds will suffice to remove the paraffin if the slide be plunged into a tube of xylol or toluol. For *thin* sections, 8 to 10 μ, it is not necessary to warm at all. The sections may be mounted direct from the xylol or the slide may be brought into a tube of alcohol to remove the solvent for staining.

Paraffin sections *can* be stained without removal of the paraffin, so that after-treatment with alcohol can be suppressed (Mayer, *Grundzüge*, p. 7), but this is only very exceptionally advantageous.

158. Recapitulation of the Paraffin Method, as recommended to be practised for small objects.—Put into a small test-tube enough oil of cedar to cover your object. On to the oil pour carefully the same quantity of absolute alcohol. Take your (already dehydrated) object and put it carefully into the alcohol. Leave it until it has sunk to the bottom of the cedar oil. Wait till the refraction lines, § 123, have vanished. Then put it into paraffin kept at melting-point in a watch glass. Let the paraffin be of the *very lowest melting-point* that will give sufficiently thin sections. After a time change the paraffin (*i. e.* put the object into a fresh watch glass with clean paraffin) once, or twice if the object be at all large. As soon as the object is thoroughly soaked with paraffin float the watch glass on cold water. When cool, cut out a block of paraffin containing the object, and fix it with a heated needle on a cone of paraffin already mounted on the object-carrier of the microtome.

Trim and orient the block and knife according to circumstances, as directed, §§ 151, 152. Cut the sections, singly if desired, or for convenience in ribbons. Collodionise if necessary, § 154. When cut always flatten and expand on water, § 156. Fix them in serial order on a slide by one of the methods given in Chapter X, the water method by preference if they have to be stained. Remove the paraffin with xylol. Stain, or mount direct.

Paraffin Masses.

159. Pure Paraffin.—It is now almost universally admitted that pure paraffin is superior for ordinary work to any of the many mixtures with wax and the like that used to be recommended. *A paraffin melting at* 50° C. is that which in my experience gives the best results *so long as the temperature of the laboratory is between* 15° *and* 17° C. For higher temperatures a harder paraffin is required, and for lower temperatures a softer one.

Many workers of undoubted competence prefer masses somewhat harder than those recommended, viz. of melting-points varying between 50° and 55° C. for the normal temperature of the laboratory; and others recommend masses melting at 60° C. or higher.

So, for instance, Heidenhain (58°), Apáthy (55°), Rabl (56°), Mayer (58° to 60° in summer; in winter about 56°, but never less than 50°). Mayer points out (*Grundzüge*, p. 101) that at Naples the temperature during five months of the summer and autumn is over 22° C. in the laboratory, sometimes over 30°. Temperatures such as these are seldom realised in the British Isles, and whilst I quite admit that the hard paraffin employed by Mayer may have its *raison d'être* for Naples, I hold that for that very reason it is in general unnecessarily hard for cooler climates.

My recommendation of a relatively soft paraffin refers to work with the Thoma sliding microtome. Microtomes with *fixed* knives, such as the Cambridge, the Minot, or the Reinhold-Giltay, will give good results, so far as cutting is concerned, with much harder paraffin, and, in fact, require such.

For thin sections a harder paraffin is required than for thick ones; and the thinner the sections, the harder should the paraffin be.

Hard objects require a harder paraffin than soft ones.

Paraffin had better be obtained from Grübler, or one of the known dealers in microscopic reagents. Brass (*Zeit. wiss. Mik.*, ii, 1885, p. 300) recommends such as has been kept for some years, as it has less tendency to crystallise than new paraffin.

Paraffin of various melting-points is easily found in commerce. Intermediate sorts may be made by mixing hard and soft paraffin. I find that two parts of paraffin melting at 50° with one of paraffin melting at 36° C. give a mass melting at 48° C., and a mixture of one part of that melting at 53° with one part of that melting at 45° gives a mass melting at 50° C.

According to E. Burchardt (*Jena. Zeit. Naturw.*, xxxiv, 1900, p. 719) *mixtures* of paraffins of different melting points give better results than an unmixed paraffin of the same melting point as the mixture. He recommends 10 parts of 40° paraffin + 1 of 45° + 1 of 52° + 1 of 58° + 6 of 60°. I think there is something in this.

160. Overheated Paraffin.—Graf Spee (*Zeit. wiss. Mik.*, ii, 1885, p. 8) takes paraffin of about 50° C. melting-point and heats it in a porcelain capsule by means of a spirit lamp. After a time disagreeable white vapours are given off, and the mass shrinks a little. This result is arrived at in from one to six hours. The mass then becomes brownish-yellow, and after cooling shows an unctuous or soapy surface on being cut. The melting point will be found to have risen several degrees. This mass may be obtained ready prepared from Grübler. The object of this preparation is to make the mass stickier, in view of cutting ribbons.

For mixtures of paraffin with other substances, none of which I consider to offer any advantage, see previous editions. I am surprised to see that Van Walsem (*Verh. Akad. Wetensch. Amsterdam*, 1899, p. 132) still recommends the addition of 5 per cent. of yellow wax to paraffin of 52° to 57° melting point (for large sections of central nervous system).

JOHNSTON (*Journ. Appl. Micr.*, vi, 1903, p. 2662) adds 1 per cent. of india-rubber in very small pieces, dissolved by heating to 100° C. for twenty-four hours, or several days to 60° C. Clear with xylol. For very brittle objects.

161. Soap Masses.—These have never been much used, and are now entirely discarded in favour of paraffin. But see previous editions, or the papers of PÖLZAM (*Morph. Jahrb.*, iii, 1877, p. 558, from Salensky's paper on the gemmation of Salpa, *loc. cit.*); KADYI (*Zool. Anz.*, 1879, vol. ii, p. 477); DÖLLKEN (*Zeit. wiss. Mik.*, xiv, 1897, p. 32). A formula of FISCHER is given *post*, under TREMATODES.

Gelatin Masses.

162. Gelatin Imbedding is a method that has the advantage of being applicable to tissues that have not been *in the least degree dehydrated.*

The *modus operandi* is, on the whole, the same as for other fusion masses, with the difference that the objects are prepared by saturation with *water* instead of alcohol or a clearing agent. After the cooling of the mass it may sometimes be cut at once, but it is generally necessary to harden it. This may be done by treatment for a few minutes with absolute alcohol (KAISER), or for a few days with 90 per cent. alcohol (KLEBS) or chromic acid (KLEBS) or formaldehyde (NICOLAS), or it may be frozen (SOLLAS).

The mass can be removed from the sections by means of warm water.

APÁTHY (*Mitth. zool. Stat. Neapel*, xii, 1897, p. 718) saturates objects with thin glycerin-gelatin, allows the water to evaporate from it in a desiccator kept just at the melting temperature of the mass, imbeds in metal squares, § 144, hardens in absolute alcohol, and cuts under the same.

163. KLEB'S **Gelatin (Glycerin Jelly)** *Arch. mik. Anat.*, v, 1869, p. 165).—A concentrated solution of isinglass is mixed with half its volume of glycerin.

164. KAISER'S **Gelatin** (*Bot. Centralb.*, i, 1880, p. 25).—One part by weight of the finest French gelatin is left for about two hours in 6 parts by weight of water; 7 parts of glycerin are added, and for every 100 grms. of the mixture 1 grm. of concentrated carbolic acid. The whole is warmed for ten to fifteen minutes, stirring all the while, until the whole of the flakes produced by the carbolic acid have disappeared. Filter whilst warm through the finest spun glass, which has been previously washed in water and laid whilst wet in the funnel.

165. GERLACH'S **Gelatin** (GERLACH, *Unters. a. d. Anat. Inst. Erlangen,* 1884; *Journ. Roy. Mic. Soc.,* 1885, p. 541).—Take gelatin, 40 grms.; saturated solution of arsenious acid, 200 c.c.; glycerin, 120 c.c. Clarify with white of egg. The mass may be kept for years in a well-stoppered bottle. The objects to be prepared for imbedding by a bath of one third glycerin.

166. BRUNOTTI'S **Gold Gelatin Mass** (*Journ. de Botan.,* vi, 1892, p. 194; *Journ. Roy. Mic. Soc.,* 1892, p. 706).—Twenty grms. gelatin dissolved with heat in 200 c.c. distilled water, and 30 to 40 c.c. of glacial acetic acid with 1 grm. corrosive sublimate added after filtering. At the temperature of 15° C. the mass has the consistence of a thick syrup. Objects are prepared by soaking in some of the mass diluted with two to three vols. of water, then imbedded in the undiluted mass. The mass is then hardened in spirit or bichromate of potash, picric acid, or the like. No heat at all is required in this process. This mass is particularly recommended by JANSSENS, *La Cellule,* xix, 1901, p. 33.

167. NICOLAS'S **Method** (*Bibliogr. Anat.,* Paris, 3 année, 1896, p. 274).—Preparations are first soaked for one or two days in a 3 per cent. to 4 per cent. aqueous solution of gelatin kept at 25° C., then for the same time in a 10 per cent. solution, and then for two or three days more in a 20 per cent. to 25 per cent. solution containing 8 per cent. to 10 per cent. of glycerin and kept at 35° C. They are then imbedded in some of the same mass in paper trays, and as soon as the gelatin has set are thrown into a 5 per cent. solution of formaldehyde (formol 1 part, water 7). After a few days therein the gelatin has become hard and insoluble, and may be cut or preserved for months in weak formol solution, or dilute alcohol or glycerin, or even in pure water. The mass cuts like celloidin, but unfortunately takes stains strongly. The sections must be very carefully and gradually passed through the successive alcohols for dehydration, as they curl up very easily. They, however, flatten out at once on being brought from absolute alcohol into cresylol, and may thence be mounted in balsam. To mount in glycerin is of course easy.

CHAPTER IX.

COLLODION (CELLOIDIN) AND OTHER IMBEDDING METHODS.

168. Introduction.—Collodion (or celloidin) massses do not require the employment of heat. They do not require that the objects should be cleared before imbedding, and that is an advantage in the case of very large objects. They are more or less transparent, which facilitates the orientation of the object. And they are specially indicated for very large objects, for the soaking in collodion being quite inoffensive to the most delicate elements may be prolonged if necessary for weeks, thus ensuring the harmless penetration of objects that would be literally cooked if they were submitted to a paraffin bath of like duration. Lastly, the mass being quite transparent after mounting, it is not necessary to remove it from the sections before staining and mounting them; it may remain, and fulfil the function of an admirable support to the tissues, holding in their places brittle or detached elements that without that help would fall to pieces and be lost.

There are disadvantages. One is that the process is a very long one; as usually practised, the collodion process requires some three days for the imbedding of an object that can be imbedded in paraffin in an hour (though the time may be greatly abridged by Gilson's rapid process given below). Another is that it is impossible to obtain with celloidin sections as thin as those furnished by paraffin; the lowest limit I have been able to attain to is 7 μ, which for some work is not sufficient. Other workers seem to have obtained thinner ones; but at any rate this cannot be done without difficulty.

In the *older celloidin method* the mass is cut *wet, before clearing.* I strongly recommend the more recently introduced practice of *clearing before cutting,* and *cutting dry* as described in §§ 181-183.

169. Collodion, Celloidin, and Photoxylin.—The collodion method is due to DUVAL (*Journ. de l'Anat.*, 1879, p. 185).

Celloidin, recommended later on by MERKEL and SCHIEFFERDECKER (*Arch. Anat. Phys.*, 1882, p. 200), is merely a patent collodion. It may be obtained from GRÜBLER, or the other dealers in histological reagents. It is sent out in the form of tablets. These tablets may, if desired, be dissolved at once in ether, or a mixture of ether and alcohol, to make a collodion of any desired strength. But it is better, as recommended by APÁTHY, to cut them up into thin shavings, which should be allowed to dry in the air until they become yellow, transparent, and of a horny consistency, and that these be then dissolved in alcohol and ether (sulphuric, free from acid). The solutions thus prepared are *free from the excess of water* that is present in the undried celloidin, and give after hardening a mass that is *more transparent* and of a better consistency for cutting (*Zeit. wiss. Mik.*, vi, 1889, p. 164).

Imbedding masses of excellent quality can be prepared with ordinary collodion, but celloidin furnishes more readily solutions of known concentration. Otherwise there is but little to choose between the two, and therefore in this work the terms collodion and celloidin are used indifferently.

According to UNNA (*Monatschr. p. Dermatol.*, xxx, 1900, pp. 422 and 476; *Zeit. wiss. Mik.*, xviii, 1901, p. 32) a more inelastic, and therefore better, mass is obtained by adding to celloidin 2 per cent. of oil of turpentine, stearate of soda, or (best of all) castor oil. Celloidin with this addition has been put on the market under the name of "Celloidinum inelasticum," by the *Chemische Fabrik vorm. E. Schering*, in Berlin.

Photoxylin (KRYSINSKY, VIRCHOW'S *Archiv*, cviii, 1887, p. 217; BUSSE, *Zeit. wiss. Mik.*, ix, 1892, p. 47) is a dry substance, of the aspect of cotton wool, and chemically nearly related to celloidin. It can be obtained from GRÜBLER. It gives a clear solution in a mixture of equal parts of ether and absolute alcohol, and should be used in exactly the same way as celloidin. It has the advantage of affording a mass which after hardening in 85 per cent. alcohol remains perfectly *transparent.* But celloidin or common collodion also gives perfectly transparent *masses if cleared in bulk* as I recommend should be done (§§ 181–183); so that there is no advantage on this head, unless it be desired to proceed in the old way. Some writers say that it gives a better consistency, but others deny this (APÁTHY, *e. g.*).

Tschernischeff (*Zeit. wiss. Mik.*, xvii, 1900, p. 449) recommends *Colloxylin* (10 grms. dissolved in 10 grms. of eugenol or clove oil, with the addition of 50 c.c. of ether and 1 of absolute alcohol).

Marpmann (*Zeit. angew. Mik.*, ix, 1903, p. 14; *Journ. Roy. Mic. Soc.*, 1903, p. 558) recommends *celluloid* dissolved in about 10 vols. of acetone.

The Older Celloidin Method.

170. Preparation of Objects.—The objects must first be *very thoroughly* dehydrated with absolute alcohol. They are then soaked till thoroughly penetrated in ether, or, which is better, in a mixture of ether and absolute alcohol. Duval (*loc. cit.*) takes for this purpose a mixture of ten parts of ether to one of alcohol; Schiefferdecker (and the majority of workers) a mixture of equal parts of ether and alcohol; Tubby (in *Nature*, November 17th, 1892, p. 51) advises a mixture of four parts of ether and one of alcohol. But the point is one of no great importance. Fish advises acetone, see next §.

This stage may be omitted if the objects are of a sufficiently permeable nature, and they may be brought direct from alcohol into the collodion bath.

171. The Collodion Bath.—The secret of success here is to infiltrate the objects first with thin solutions, then with the definitive thick one. (A thin solution may be taken to mean one containing from 4 to 6 per cent. of celloidin [dried as described in § 169]; a thick solution, one containing 10 to 12 per cent.)

If collodion be taken, the thin solutions may be made by diluting it with ether. If photoxylin or celloidin be taken, the solutions are made in a mixture of ether and absolute alcohol in equal parts.

The dried celloidin shavings dissolve very slowly in the mixture. Elschnig (*Zeit. wiss. Mik.*, x, 1893, p. 443) states that solution is obtained much quicker if the shavings be first allowed to swell up for twenty-four hours in the necessary quantity of absolute alcohol, and the ether be added afterwards. On trial it seems to me that this is so.

Busse (*op. cit.*, ix, 1892, p. 47) gives the following proportions for the successive baths :—No. 1, 10 parts by weight of photoxylin or perfectly dried celloidin to 150 parts of the ether and alcohol mixture : No. 2, 10 parts of photoxylin or celloidin to 105 of the mixture : No. 3, 10 parts to 80 of the mixture (already-used solution may be employed for the first bath).

I generally use only two solutions : one weak one, and one strong one corresponding approximately to Busse's No. 2. His No. 3 is so thick that excessive time is required to obtain penetration by it.

Fish (*Journ. Applied Microscop.*, ii, 1899, p. 323) first infiltrates with *acetone* (which he says may be used as a fixing and dehydrating agent at the same time), then with a 4 per cent. solution of pyroxylin (gun cotton) in acetone; and, lastly, in an 8 per cent. acetone solution of the same. See for other solutions § 183.

The objects ought to remain in the first bath until very thoroughly penetrated ;—days, even for small objects,—weeks or months for large ones (human embryos of from six to twelve weeks, for instance). If the object contain cavities, these should be opened to ensure their being filled by the mass.

When the object is duly penetrated by the thin solution, or solutions, if more than one have been employed, it should be brought into the thickest one. This may be done (as first described in this work, 1st edit., 1885, p. 194) by allowing the thin solution to concentrate slowly (the stopper of the containing vessel being raised, for instance, by means of a piece of paper placed under it), and making up the loss from evaporation with thick solution.

Apáthy (*Mikrotechnik*, p. 121) holds that it is preferable to transfer to fresh thick solution, as he finds that a better consistency after hardening is thus obtained.

172. Imbedding.—If the object is such that it can be fixed, by gumming or otherwise, to the holder of the microtome without the intervention of any specially shaped mass of collodion around it, and if the presence of such a mass be not required in the interest of the orientation of the object, or of the production of continuous series of sections, or of very thin sections, no special imbedding is necessary, and as soon as the objects are duly penetrated by the thick solution you may proceed to the hardening part of the process. In other words, it is waste of time to get the object into a special block of collodion if that is not rendered desirable for the reasons above mentioned. But for fine and regular work I hold that it is necessary.

In that case the objects must at this stage, if it has not been done before, be *imbedded*—that is, arranged in position in the thick collodion in the receptacle in which they are to be hardened. For the usual manipulations see § 144. I recommend the paper thimbles or cylindrical trays, Fig. 2, as being very convenient for collodion imbedding. The bottoms, however, should be made of soft wood in preference to cork, see § 177. The box should be prepared for the reception of the object by pouring into it a drop of collodion, which is allowed to dry. The object of this is to prevent bubbles coming up through the wood or cork and lodging in the mass.

Objects may also be imbedded on a piece of pith or leather, which should also be prepared with a layer of dry collodion.

Watch glasses, deep porcelain water-colour moulds, and the like, also make convenient imbedding receptacles. Care should be taken to have them perfectly *dry*.

It not infrequently happens that during these manipulations bubbles make their appearance in the mass. Before proceeding with the hardening these should be got rid of. This may be done by exposing the whole for an hour or two to the vapour of ether in a desiccator or other well-closed vessel. Care should be taken that the ether (which may be poured on the bottom of the vessel) does not wet the mass (Busse, *Zeit. wiss. Mik.*, viii, 1892, p. 467).

173. Orientation of the Objects.—Celloidin being more or less transparent, it is seldom necessary to recur to special aids to orientation.

Apáthy (*Zeit. wiss. Mik.*, v, 1888, p. 47) arranges objects on a small rectangular plate of gelatin, placed on the bottom of the imbedding-recipient. The gelatin is turned out with the mass after hardening, and cut with it. The edges of the gelatin form good orientation lines.

Halle and Born (*Zeit. wiss. Mik.*, xii, 1896, p. 364) use plates of hardened white of egg, in which a shallow furrow for the reception of the objects has been cut by means of a special instrument. See also § 149.

For the complicated method of EYCLESHYMER (*Amer. Nat.*, xxvi, 1892, p. 354) see *previous editions.*

See also the article "Plastische Rekonstruction" in the *Encycl. mik. Technik.*

174. Hardening, Preliminary.—This is logically the next step, but as a matter of fact is frequently begun before. For the different processes of the collodion method so run into one another that it is difficult to assign natural lines of demarcation between them.

The objects being imbedded, the treatment should be as follows:—The receptacles or supports are set with the mass under a glass shade, allowing of just enough communication with the air to set up a slow evaporation. Or porcelain moulds or small dishes may be covered with a lightly-fitting cover. As soon as the added thick collodion (of which only just enough to cover the object should have been taken) has so far sunk down that the object begins to lie dry, fresh thick solution is added, and the whole is left as before. (If the first layer of collodion has become too dry, it should be moistened with a drop of ether before adding the fresh collodion.) Provision should be again made for slow evaporation, either in one of the ways above indicated, or—which is perhaps better—by setting the objects under a *hermetically* fitting bell-jar, which is lifted for a few seconds only once or twice a day. I have frequently found it advantageous to set the objects under a bell-jar, together with a dish containing alcohol, so that the evaporation is gone through in an *atmosphere of alcohol.* This is especially indicated for very large objects. The whole process of adding fresh collodion and placing the objects under the required conditions of evaporation is repeated every few hours for, if need be, two or three days.

When the mass has attained a consistency such that the ball of a finger (*not* the nail) no longer leaves an impress on it, it should be scooped out of the dish or mould, or have the paper removed if it has been imbedded in paper, and be submitted to the next stage of the hardening process. (If the mass is found to be not quite hard enough to come away safely, it should be put for a day or two into weak alcohol, 30 to 70 per cent.)

175. Hardening, Definitive.—Several methods are available for the definitive hardening process. One of these is the *chloroform* method, due to VIALLANES (*Rech. sur l'Hist. et le Dév des Insectes*, 1883, p. 129). I recommend this method for *small* objects because I find it much more rapid than the alcohol method, whilst giving at least as good a consistency to the mass. (For *large* objects the method is said by some writers to be inferior to the alcohol method, because the rapid hardening of the external layers is an obstacle to the diffusion necessary to the hardening of the inner layers.)

It consists in bringing the objects into *chloroform*. Under the influence of this reagent the collodion coagulates rapidly into a mass, having the consistency of wax. In some cases a few *hours'* immersion is sufficient to give the requisite consistence. In no case have my specimens required more than three days. The collodion frequently becomes opaque on being put into the chloroform, but regains its transparency after a time.

Small objects may be hardened by chloroform *without preliminary hardening by evaporation*. All that is necessary is to expose the mass to the air for a few seconds until a membrane has formed on it, and then bring it into chloroform. If the mass is in a test-tube this may be filled up with chloroform and left for two or three days if need be. By this time the collodion mass will be considerably hardened, and also somewhat shrunk, so that it can be shaken out of the tube. It is then brought into fresh chloroform in a larger vessel, where it remains for a few more days until it is ready for cutting. But sufficient hardening is sometimes obtained in a few hours.

Good chloroform is a necessity.

The above processes are excellent, but I regard them as primitive forms of the chloroform method. I now almost always harden in *vapour of chloroform*. All that is necessary is to put the liquid mass (after having removed bubbles as directed in § 172) with its recipient into a desiccator on the bottom of which a few drops of chloroform have been poured.

The action is very rapid, and the final consistency of the mass at least equal to that obtained by the best alcohol hardening. We shall revert to this subject, § 183.

The more commonly employed hardening method is the *alcohol* method. The objects are thrown into alcohol and left there until they have attained the right consistency (one day to several weeks). The bottle or other vessel containing the alcohol *ought not to be tightly closed, but should be left at least partly open.*

The strength of the alcohol is a point on which the practice of different writers differs greatly. The question may now be considered to be finally settled by experiments specially directed to the clearing up of this point, made by BUSSE (*Zeit. f. wiss. Mik.*, ix, 1, 1892, p. 49), and which I have repeated and confirmed. BUSSE finds that *alcohol of about* 85 *per cent.* is the best, *both as regards the cutting consistency and the transparency* of the mass. Care must be taken to keep masses hardened in this grade of alcohol moist whilst cutting, as they dry by evaporation very quickly.

Some workers use lower grades, 70 to 80 per cent., or even lower. APÁTHY (*Mikrotechnik*, p. 185) mentions "glycerin-alcohol," but without giving details. BLUM (*Anat. Ans.*, xi, 1896, p. 724) mentions "weak spirit with formol added to it," saying that formol hardens celloidin.

Lastly, the mass may be *frozen.* After preliminary hardening by alcohol, it is soaked for a few hours in water, in order to get rid of the greater part of the alcohol (the alcohol should not be removed entirely, or the mass may freeze too hard). It is then dipped for a few moments into gum mucilage in order to make it adhere to the freezing plate, and is frozen. If the mass have frozen too hard, cut with a knife warmed with warm water.

A paper has been written by FLORMAN (*Zeit. wiss. Mik.*, vi, 1889, p. 184) to recommend that the definitive hardening should be done without the aid of alcohol or chloroform, by simply cutting out the blocks, turning them over, and carefully continuing the evaporation process in the way described above. I described this process myself in the first edition of this work. No doubt the author is right in claiming for it a greater degree of hardening of the mass; but I doubt whether it is possible to carry the hardening much beyond the point attained by the chloroform or alcohol method without incurring a very undesirable degree of shrinkage.

176. Preservation.—The hardened blocks of collodion may be preserved till wanted in weak alcohol (70 per cent.). They may also be preserved dry by dipping them into melted paraffin (APÁTHY, *Zeit. wiss. Mik.*, v, 1888, p. 45), or, after rinsing the water, in glycerine-jelly, which may be removed with warm water before cutting (APÁTHY, *Mitth. Zool. Stat. Neapel*, xii, 1897, p. 372).

Reference numbers may be written with a soft lead pencil on the bottom of the paper trays, or with a yellow oil pencil on the bottom of the watch glasses in which the objects are imbedded. On removal of the paper from the collodion after hardening, the numbers will be found impressed on the collodion.

177. Cutting.—If the object *has not been stained* before imbedding, it may form so transparent a mass with the collodion that the arrangement of the object and sections in the right position may be rendered very difficult. It is, therefore, well to stain the collodion lightly, just enough to make its outlines visible in the sections. This may be done by adding picric acid or other suitable colouring matter dissolved in alcohol to the collodion used for imbedding, or to the oil used for clearing.

To fix a collodion block to the microtome proceed as follows:—Take a piece of soft wood, or, for very small objects, pith, of a size and shape adapted to fit the holder of the microtome. Cover it with a layer of collodion, which you allow to dry. Take the block of collodion or the infiltrated and hardened but not imbedded object, and cut a slice off the bottom, so as to get a clean surface. Wet this surface first with absolute alcohol, then with ether (or allow it to dry); place one drop of *very thick* collodion on the prepared wood or pith and press down *tightly* on to it the wetted or dried surface of the block or object. Then throw the whole into weak (70 per cent.) alcohol for a few hours, or even less, or better into chloroform, or vapour of chloroform, for a few minutes, in order that the joint may harden.

LINDSAY JOHNSON prefers to use the cement employed by metal turners for fastening metal objects on to boxwood chucks. The exact composition of this cement varies somewhat, but an average one is—beeswax, 1 part; rosin, 2 parts. To use it you must get the block of celloidin perfectly dry at the bottom, then warm the object-holder slightly, if possible over a flame; drop on to it a few drops of melted cement, and press on to it the block of collodion, which will be firmly fixed as soon as the cement is cool—that is in a few seconds.

For objects of any considerable size it is important not to use cork for mounting on the microtome, if the object-holder be a vice; for cork bends under the pressure of the holder, and the elastic collodion bends with it, deforming the object. I have seen large embryos so deformed in this way that the sections obtained were true calottes, segments of a sphere. If the object-holder be of the cylinder type, a good cork may be used; but even then, I think, wood is safer. GAGE has recommended bits of glass cylinders. JELINEK (*Zeit. wiss. Mik.*, xi, 1894, p. 237) recommends a sort of vulcanite known as "Stabilit," which is manufactured for electrical insulation purposes. It is supplied in suitable blocks by JUNG (presumably also obtainable through GRÜBLER AND Co.). Wood is liable to swell in alcohol so that it no longer fits into the object-holder.

Sections (from such masses as have not been cleared before cutting) are cut with a knife kept abundantly wetted with alcohol (of 50 to 85 or even 95 per cent.). Some kind of drip arrangement will be found very useful here. Apáthy recommends that the knife be smeared with yellow vaseline; it cuts better, is protected from the alcohol, and the mobility of the alcohol on the blade is lessened.

The knife is set in as oblique a position as possible.

Very brittle sections may be collodionised as explained, § 154.

The sections are either brought into alcohol (of 50 to 85 or 95 per cent.) as fast as they are made; or if it be desired to mount them in series, they are treated according to one of the methods described below, in Chapter X.

Masses that have been cleared before cutting with cedar oil or the like may be cut *dry*, § 183.

178. Staining.—The sections may now be stained as desired, either loose, or mounted in series on slides or on paper as described in Chapter X. It is *not* in general necessary, nor indeed desirable, to remove the mass before staining, as it usually either remains colourless, or gives up the stain on treatment with alcohol. But some dyes stain it strongly, and are not removed with sufficient completeness by the processes of dehydration and clearing. If it be desired to employ these, the mass may be removed by treating the sections with absolute alcohol or ether.

179. Clearing and Mounting.—You may mount in glycerin without removing the mass, which remains as clear as glass in that medium.

You may mount in balsam, also, without removing the mass, which does no harm, and serves the useful purpose of holding the parts of the sections together during the manipulations. Dehydrate in alcohol of 95 or 96 per cent. (not absolute, as this attacks the collodion). NIKIFOROW (*Zeit. wiss. Mik.*, viii, 1891, p. 189) recommends a mixture of equal parts of alcohol and chloroform. Clear with a substance that does not dissolve collodion. The clearing agents most recommended are origanum oil (*Ol. Origan. Cretici*, it is said, should be taken, not *Ol. Orig. Gallici*; but see as to this reagent the remarks in § 130), bergamot oil (said to make sections shrink somewhat), oil of sandal-wood, lavender oil, oil of cedar-wood (safe and gives excellent results, but acts rather slowly), chloroform, xylol, or benzol (may make sections shrink if not well dehydrated), or Dunham's mixture of three or four parts of white oil of thyme with one part of oil of cloves. (As to oil of thyme, see also §§ 130, 131.)

FISH (*Proc. Amer. Mic. Soc.*, 1893) advises a mixture of one part of red oil of thyme with three parts of castor oil, the latter being added in order to counteract the volatility of the thyme oil. But later (June, 1895), writing to me, Dr. Fish says he has substituted the white oil of thyme for the red, and finds it an advantage in orientating. See also § 131.

Some specimens of clove oil dissolve collodion very slowly, and may be used, but I would not be understood to recommend it. The action of origanum oil varies much, according to the samples; some sorts do not clear the collodion, others dissolve it, others pucker it. MINOT (*Zeit. wiss. Mik.*, iii, 1886, p. 175) says that Dunham's mixture "clarifies the sections very readily, and softens the celloidin just enough to prevent the puckering which is so annoying with thyme alone."

Carbolic acid has been recommended. WEIGERT (*Zeit. wiss. Mik.* iii, 1866, p. 480) finds that a mixture of 3 parts of xylol with 1 part of carbolic acid (anhydrous) clears well. But it must not be used with the basic anilin stains, as it discolours them. For these anilin oil may be used with the xylol in the place of carbolic acid.

Anilin oil clears well (it will clear from 70 per cent. alcohol), but unless thoroughly removed the preparation becomes yellowish-brown. This coloration may be removed by soaking in chloroform for twenty-four hours (see VAN GIESON, *Amer. Mon. Mic. Journ.*, 1887, p. 49, or *Journ. Roy. Mic. Soc.*, 1887, p. 519, for a review of these clearing agents; see also § 139.

Beech-wood creasote has been recommended (by M. Flesch).

EYCLESHYMER (*Amer. Nat.*, xxvi, 1892, p. 354) advises a mixture of equal parts of bergamot oil, cedar oil, and carbolic acid.

For oil of cajeput see § 134; and for this and other clearers see also the paper of JORDAN quoted § 122.

The Newer Celloidin Method.

180. The Two Methods Compared.—The older celloidin method, described in the foregoing pages, is extremely lengthy and cumbrous. The operation of infiltrating the tissues with the collodion requires days or weeks. The hardening process frequently requires nearly as much time. The resulting mass has the disadvantage of being semi-opaque, at most only translucent, not transparent. It has to be cut under the surface of alcohol, or at least with constant wetting with alcohol, and with a knife kept constantly wet with alcohol. By the recent method of clearing before cutting a large part of these defects is done away with; the resultant mass is as clear as glass, thus allowing the most perfect orientation of the object; and, as I have shown (LEE et HENNEGUY, *Traité des Méthodes techniques de l'Anat. mic.*, 1896, p. 230), the mass can with advantage be cut *dry*, thereby greatly simplifying the operation of cutting. By GILSON's ingenious *Rapid Method*, the time necessary for the whole series of operations is very greatly abridged. I cannot imagine that anyone who has ever employed the new method would willingly go back to the old one.

181. The New Method, by Clearing before Cutting.—This process is due, I believe, in the first instance to E. MEYER (*Biol. Centralb.*, x, 1890, p. 508), who advised soaking blocks before cutting for twenty-four hours in glycerin. BUMPUS (*Amer. Anat.*, xxvi, 1892, p. 80) advises clearing the mass, after hardening in chloroform, with white oil of thyme or other suitable clearing agent (see § 179). The knife is wetted with the clearing oil, and the same oil is employed for covering the exposed surface of the object after each cut. Similar recommendations are made by EYCLESHYMER (*op. cit.*, pp. 354, 563), carbolic acid, or glycerin, or the mixture given § 179, being suggested for clearing; and GILSON has for a long time past adopted the practice of clearing before cutting with cedar oil, as described in the next §.

FISH (*loc. cit.*, § 179) also advocates the practice of clearing in the mass, recommending the clearing mixture there given. Similarly GAGE, *Trans. Amer. Mic. Soc.*, xvii, 1896, p. 361.

All the authors above quoted cut in the *wet* way, that is to say, with a knife wetted with the clearing liquid. I have found a great improvement in cutting *dry*, and in employing the combined hardening and clearing process of GILSON, given below.

182. GILSON'S **Rapid Process** (communicated by Professor GILSON, April, 1892).—The object is dehydrated, soaked in ether, and brought into a test-tube with collodion or thin celloidin solution. The tube is dipped into a bath of melted paraffin, and the collodion allowed to boil (which it does at a very low temperature) until it has become of a syrupy consistence. (It should be boiled down to about one third of its volume.) The mass is then turned out, mounted on a block of hardened celloidin, and the whole hardened in chloroform or in a mixture of chloroform and cedar oil for about an hour. It is then cleared in cedar oil (if hardened in pure chloroform: special clearing will not be necessary if it has been hardened in the mixture). It may now be fixed in the microtome and cut, using cedar oil to wet the knife, and cover the exposed surface of the object after each cut.

This process is very much more rapid than the old process in two ways. The celloidin bath, being given warm, is greatly abridged; small objects can be duly infiltrated in an hour, where days would be required by the old process. And the hardening is very much more rapid than hardening by alcohol, which requires at least twenty-four hours. As collodion boils at a very low temperature very little heat is required, and there is no risk of the tissues suffering on that head.

183. The Dry Cutting Method.—I recommend the following as being a further improvement. Infiltrate with collodion or celloidin either by GILSON'S process, or by soaking in the cold in the usual way, § 171. This is a much slower process, but does not take up more of the worker's time, as the specimens require no attention whilst in the bath. Imbed as usual, either directly on the holder of the microtome, or in a paper tray or a water-colour mould or the like. Harden in vapour of chloroform for from one hour (generally sufficient for small objects) to overnight. This is done by putting the object (definitively imbedded in the final thick solution, but without any preliminary hardening in the air) into a Steinach's sieve-dish or into a desiccator, on the bottom of which a teaspoonful of chloroform has been poured. (The objects may remain for months in the chloroform vapour if desired.) As soon as the mass has attained sufficient superficial hardness, it is, of course, well to turn it out of its recipient, and turn it over from time to time, in order that it may be equally exposed on all sides to the action of the vapour. When fairly hard (it is not necessary to wait till the mass has attained all the hardness of which it is susceptible) throw it into GILSON'S mixture. This should be at first a mixture of one part of chloroform with one or two parts of cedar oil. From time to time more cedar oil should be added, so as to bring the mixture up gradually to nearly pure cedar oil. As soon as the object is cleared throughout, the mass may be exposed to the air, and the rest of the chloroform will evaporate gradually. The block may now be mounted on the holder of the microtome with a drop of thick collodion, § 177, and may either be cut at once, or may be preserved indefinitely without change in a stoppered bottle. *Cut dry*, the cut surface will not dry injuriously under several hours. The cutting quality of the mass is often improved by allowing it to evaporate in the air for some hours.

The hardening may be done at once in the chloroform and cedar-wood mixture, instead of the chloroform vapour, but I find the latter process preferable, as giving a better hardening. And clearing may be done in pure cedar oil instead of the mixture, but then it will be very slow, whereas in the mixture it is extremely rapid.

Stepanow (*Zeit. wiss. Mik.*, xvii, 1900, p. 185) soaks and imbeds in a solution of celloidin in a mixture of equal parts of *ether and clove oil*, hardens in alcohol or vapour of chloroform, or in *benzol*, and cuts either wet or dry.

I fail to grasp the *raison d'être* of the modifications described by Tschernischeff, *ibid.*, p. 449.

Jordan, *ibid.*, p. 193, imbeds in a mixture of 5 parts of 8 per cent. celloidine solution with one of *oil of cedar*, hardens first in vapour of chloroform and then in a mixture of 5 parts of chloroform with one of oil of cedar, and cuts wet or dry.

184. Double Imbedding in Collodion and Paraffin.—This is sometimes, though rarely, employed for objects of which it is desired to have very thin sections, and which are too brittle to give good sections by the plain paraffin process.

Kultschitzky's **Method** (*Zeit. wiss. Mik.*, iv, 1887, p. 48).—After the collodion bath, the object is soaked in oil of origanum (*Oleum Origani vulg.*). It is then brought into a mixture of origanum oil and paraffin heated to not more than 40° C., and lastly into a bath of pure paraffin.

The mass may be preserved in the dry state, and may be cut dry.

Ryder (*Queen's Micr. Bull.*, 1887, p. 43; *Journ. Roy. Mic. Soc.*, 1888, p. 512) modified the process by substituting chloroform for the origanum oil.

Ide (*La Cellule*, vii, 1891, p. 347, and viii, 1, 1892, p. 114) imbeds in collodion in a tube by Gilson's process (§ 182); the collodion is boiled for forty minutes, then brought for fifteen minutes (this is for small objects) into chloroform heated to 30° C. containing one fourth part of paraffin dissolved in it, then for ten minutes into pure melted paraffin.

Field and Martin (*Bull. Soc. Zool. de France*, 1894, p. 48) make a solution of dried celloidin in a mixture of equal parts of absolute alcohol and toluene, of about the consistency of clove oil. This solution is saturated with paraffin, added in shavings at a temperature not exceeding 20° to 23° C. The tissues are prepared by soaking in some of the mixture of alcohol and toluene, and are then penetrated with the celloidin-paraffin solution. The mass is hardened in a saturated solution of paraffin in chloroform or in toluene, and is finally imbedded in pure paraffin in the usual way.

Stepanow imbeds in paraffin after clearing with benzol, last §.

Jordan, after imbedding as in last §, passes through a bath of paraffin dissolved in chloroform into pure paraffin.

See also the modifications of Dahlgren, *Journ. Applied Microsc.*, 1898, p. 97 (*Journ. Roy. Mic. Soc.*, 1898, p. 489); Sabussow, *Mitth. Zool. Stat. Neapel*, xii, 1896, p. 353; Meyer, *ibid.*, xiv, 1901, p. 295; Mitrophanow, *Arch. Zool. Expér.* [3], 3, 1896, p. 617).

Other Cold Masses.

185. Joliet's Gum and Glycerin Method—(*Arch. Zool. Expér. et Gén.*, x, 1882, p. xliii).—Pure gum arabic dissolved in water to the consistency of a thick syrup. (Solutions of gum sold under the name of strong white liquid glue [*"colle forte blanche liquide à froid"*] may also be used; they have the advantage of having a uniform consistency.*) Pour a little of the solution into a watch glass, so as not quite to fill it, add from 6 to 10 drops of pure glycerin, stir until thoroughly mixed. In the winter or in rainy weather less glycerin should be taken than in the summer or dry weather.

The object is imbedded in the mass in the watch glass, and the whole left to dry for from one to four days. When it has assumed a cartilaginous consistency, a block containing the object is cut out, turned over, and allowed to dry again until wanted for use. A stove, or the sun, may be employed for drying, but it is best to dry slowly at the normal temperature.

This process may render service occasionally in the study of extremely watery organisms, such as *Salpa*, or the Ctenophora.

186. STRICKER'S **Gum Method** (*Hdb. d. Gewebel.*, p. xxiv).—A concentrated solution of gum arabic. The object is imbedded in the gum in a paper case. The whole is thrown into alcohol, and after two or three days may be cut. The alcohol should be of about 80 per cent. (MAYER).

I have seen masses of sufficiently good consistency prepared by this simple method.

187. ROBERTSON'S **Grape-sugar Method**, see *Journ. of Anat. and Physiol.*, xxiv, 1890, p. 230.

188. HYATT'S **Shellac Method**, see *Am. M. Mic. Journ.*, i, 1880, p. 8; *Journ. Roy. Mic. Soc.*, iii, 1880, p. 320.

This process is merely intended for the purpose of making sections through hard chitinous organs consisting of several pieces, such as stings and ovipositors, retaining all the parts in their natural positions.

189. BRUNOTTI'S **Cold Gelatin Mass** has been given, § 166.

Masses for Grinding Sections.†

190. G. VON KOCH'S **Copal Method** (*Zool. Anz.*, i, 1878, p. 36).—Small pieces of the object are stained in bulk and

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* It is highly probable that these commercial preparations contain gelatin and perhaps some other gum besides gum arabic.

† For the manipulations of section-grinding, see the Treatises on the Microscope, particularly CARPENTER'S *The Microscope and its Revelations* (J. and A. Churchill, London).

dehydrated with alcohol. A thin solution of copal in chloroform is prepared by triturating small fragments of copal in a mortar with fine sand, pouring on chloroform to the powder thus obtained, and filtering. The objects are brought into a capsule filled with the copal solution. The solution is now slowly evaporated by gently heating the capsule on a tile by means of a common night-light placed beneath it. As soon as the solution is so far concentrated as to draw out into threads that are brittle after cooling, the objects are removed from the capsule and placed to dry for a few days on the tile, in order that they may more quickly become hard. When they have attained such a degree of hardness that they cannot be indented by a finger-nail, sections are cut from them by means of a fine saw. The sections are rubbed down even and smooth on one side with a hone, and cemented, with this side downwards, to a slide, by means either of Canada balsam or copal solution. The slide is put away for a few days more on the warmed tile. As soon as the cement is perfectly hard the sections are rubbed down on a grindstone, and then on a hone, to the requisite thinness and polish, washed with water, and mounted in balsam.

The process may be varied by imbedding the objects unstained, removing the copal from the sections by soaking in chloroform, decalcifying them if necessary, and then staining.

It is sometimes a good plan, after removing the copal, to cement a section to a slide by means of hard Canada balsam, then decalcify cautiously the exposed half of the specimen, wash, and stain it. In this way von Koch was able to demonstrate the most delicate lamellæ of connective tissue, in *Isis elongata.*

This method was invented in order to enable the hard and soft parts of corals to be studied in their natural relations, and is valuable for this and similar purposes.

191. EHRENBAUM'S **Colophonium and Wax Method** (*Zeit. wiss. Mik.*, 1884, p. 414).—Ehrenbaum recommends a mass consisting of ten parts of colophonium to one of wax. The addition of wax makes the mass less brittle. Sections are obtained by grinding in the usual way. The mass is removed from them by means of turpentine followed by chloroform.

192. Johnstone-Lavis and Vosmaer's **Balsam Method** (*Journ. Roy. Mic. Soc.*, 1887, p. 200).—Alcohol material is carefully and gradually saturated, first with benzol, and then with thin and thick solution of benzol-balsam. It is then dried for a day in the air and for several days more in a hot-air bath. When hard it is ground in the usual way. For further details and figure of the drying-stove see the original, which claims for the method several advantages over that of von Koch.

193. Weil's **Canada Balsam Method** (*Zeit. wiss. Mik.*, v, 1888, p. 200).

194. Giesbrecht's **Shellac Method.**—For hard parts only, spines of *Echinus*, shell, etc., see *Morph. Jahrb.*, vi, 1880, p. 95, or the abstract in Lee und Mayer, *Grundzüge*, etc.

Congelation Masses.

195. The Freezing Method.—Fresh tissues may be, and are, frequently frozen without being included in any mass, and in certain cases very satisfactory sections can be obtained in this manner. But the *formation of ice crystals* frequently causes tearing of delicate elements, and it is better to infiltrate the tissues with a mass that does not crystallise in the freezing mixture, but becomes hard and tough. Hamilton (*Journ. of Anat. and Phys.*, xii, 1878, p. 254) soaked tissues in syrup of a particular strength, viz. double refined sugar, 2 ounces; water, 1 fluid ounce; then washed the superfluous syrup from the surface, and put into ordinary gum mucilage for an hour or so, and then imbedded in the freezing microtome with mucilage in the usual way.

196. Gum and Syrup Congelation Mass (Cole, *Methods of Microscopical Research*, 1884, p. xxxix; *Journ. Roy. Mic. Soc.* [N.S.], iv, 1884, p. 318).—Gum mucilage (B. P.), 5 parts; syrup, 3 parts. (For brain and spinal cord, retina, and all tissues liable to come in pieces put 4 parts of syrup to five of gum.) Add 5 grains of pure carbolic acid to each ounce of the medium.

(Gum mucilage [B.P.] is made by dissolving 4 ounces of picked gum acacia in 6 ounces of water.

The syrup is made by dissolving 1 pound of loaf sugar in 1 pint of water and boiling.)

This medium is employed for soaking tissues previous to freezing.

The freezing is conducted as follows:—The gum and syrup is removed from the *outside* of the object by means of a cloth; the spray is set going and a little gum mucilage painted on the freezing plate; the object is placed on this and surrounded with gum mucilage; it is thus saturated with gum and syrup, but surrounded when being frozen with mucilage only. This combination prevents the sections from curling up on the one hand, or splintering from being too hard frozen on the other. Should freezing have been carried too far, wait for a few seconds.

197. Dextrin Congelation Mass (Webb, *The Microscope*, ix, 1890, p. 344; *Journ. Roy. Mic. Soc.*, 1890, p. 113).—Thick solution of dextrin in solution of carbolic acid in water (1 in 40).

198. Gelatin (Sollas, *Quart. Journ. Mic. Soc.*, xxiv, 1884, pp. 163, 164). **Gum Gelatin** (Jacobs, *Amer. Natural.*, 1885, p. 734). **White of Egg** (Rollett, *Denkschr. math. naturw. Kl. k. Acad. Wiss. Wien*, 1885; *Zeit. wiss. Mik.*, 1886, p. 92).—Small portions of tissue brought in the white of a freshly laid egg on to the freezing stage, frozen and cut. **Oil of Aniseed** (Kuhne, *Centralb. f. Bakteriol.*, xii, 1892, p. 28; *Journ Roy. Mic. Soc.*, 1892, p. 706; V. A. Moore, *Amer. Mon. Mic. Journ.*, 1894, p. 373; *Journ. Roy. Mic. Soc.*, 1895, p. 247). **Anethol** (anise camphor), Stepanow, *Zeit. wiss. Mik.*, xvii, 1900, p. 181.

For details of these see previous editions.

For Döllken's method of solidifying formol by means of resorcin, see *Zeit. wiss. Mik.*, xiv, 1, 1897, p. 33.

CHAPTER X.

SERIAL SECTION MOUNTING.

199. Choice of a Method.—I recommend the following:—For *general* work with paraffin sections, the combined water and albumen method, § 202. For very delicate work, the water method. For collodion sections, the albumen method, or the oil of bergamot method.

Methods for Paraffin Sections.

200. The Water or Desiccation Method.—GAULE (*Arch. Anat. Phys., Phys. Abth.*, 1881, p. 156); SUCHANNEK (*Zeit. wiss. Mik.*, vii, 1891, p. 464); GULLAND (*Journ. Anat. and Phys.*, xxvi, 1891, p. 56); SCHIEFFERDECKER (*Zeit. wiss. Mik.*, ix, 1892, p. 202); HEIDENHAIN (*Kern und Protoplasma*, p. 114); NUSBAUM (*Anat. Anz.*, xii, 2, 1896, p. 52); MAYER in the *Grundzüge*, LEE und MAYER, 1898, p. 113; DE GROOT (*Zeit. wiss. Mik.*, xv, 1898, p. 62), and others (some irrational variations have been suppressed).—The principle of this method is that the sections are made to adhere to the slide without the intervention of any cementing substance, being brought into intimate contact with the glass by being slowly drawn down by the evaporation of a layer of water on which they are floated. It is now practised, with unessential variations, as follows:

(*a*) For sections that are *large and not numerous.* The sections are flattened out on water by one or other of the processes described in § 156. The slide is then drained and put away to dry until every trace of water has completely evaporated away from under the sections. This drying may be performed at the temperature of the laboratory, in which case many hours will be necessary (to be safe it will generally be necessary to leave the sections overnight). Or it may be performed in a stove or on a water-bath at a temperature a few degrees *below* the melting-point of the paraffin (best not above 40° C.), in which case fixation will be much more rapid, large thin sections being often sufficiently fixed in an hour, though thick ones will require half a dozen hours or more. *The paraffin must not be allowed to melt before the sections are perfectly dry,* the sections are sure to become detached if it does. Perfectly dry sections have a certain brilliant transparent look that is easily recognisable. As soon as dry they are perfectly fixed, and the paraffin may be removed and they may be treated with any desired liquids without more risk of their falling off than is the case with any other mode of fixation. To remove the paraffin all that is requisite is to put the slide into a tube of xylol or other good solvent, which in a few seconds, or minutes at most, removes the paraffin perfectly. Most workers first melt the paraffin, but I find this is not necessary.

(*b*) For series of *numerous small sections.* Clean a slide perfectly, so that water will spread on it without any tendency to run into drops (see below). Breathe on it, and with a brush draw on it a streak of water as wide as the sections and a little longer than the first row of sections that it is intended to mount. With a dry brush arrange the first row of sections (which may be either loose ones or a length of a ribbon) on this streak. Breathe on the slide again, draw on it another streak of water under the first one and arrange the next row of sections on it, and so on until the slide is full. Then breathe on the slide again, and with the brush add a drop of water at each end of each row of sections, so as to enable them to expand freely; then warm the slide so as to flatten out the sections, taking care *not to melt the paraffin.* Some persons do this by holding it over a small flame for a few seconds. I prefer to keep a slab of thickish glass in the drawer of the water-bath, so as to have it warm, and lay the slide on it, watching the flattening of the sections through a lens if necessary. As soon as they are perfectly flat, draw off the excess of water from one corner of the mount with a dry brush, and put aside to dry as before (*a*).

In order to success in this method it is absolutely essential that the sections be perfectly expanded and come into close contact with the slide at all points. And to ensure this it is necessary that the slide should be perfectly *free from grease,* so that the water may wet it equally everywhere. The test for this is, firstly, to breathe on the slide; the moisture from the breath should condense on it evenly all over, and disappear evenly. Secondly, streaks of water drawn on it with a brush should not run. To obtain a slide that will fulfil these conditions, proceed as follows :—

After slides have been cleaned by one of the processes given in the Appendix, **Cleaning Slides and Covers,** they should be rinsed with distilled water and preserved in 90 per cent. alcohol, from which they should be removed with forceps when required for use—not with the fingers—then simply drained, or wiped with a very clean cloth. If now a slide will not stand the breath test, place a drop of water on it and rub it in thoroughly with a damp cloth and try again. If this does not suffice, take a turn of a corner of the cloth round a finger and rub it with a piece of chalk, then damp the cloth and rub the slide with it, finishing up with a clean part of the cloth and clean water (De Groot, *loc. cit. supra*). If after performing this operation twice the slide still refuses to take the water properly, it should be rejected as incorrigible; for there are apparently some sorts of glass that can never be got to wet thoroughly.

Tap water seems preferable to distilled water; it seems to spread better and give a stronger adhesion. Nusbaum adds a trace of gum arabic (one or two drops of mucilage to a glass of water); Apáthy (*Microtechnik*, p. 126) adds 1 per cent. of Mayer's albumen (§ 201); and Henneguy (*Leçons sur la Cellule*, 1896, p. 62) takes a 1:5000 solution of gelatin, with a trace of bichromate of potash, added just before using, and dries the slides exposed to light. Similarly, Burchardt (*Jena Zeit.*, xxxiv, 1900, p. 719).

Some workers have used alcohol (50 per cent. or 70 per cent.) instead of water; but this I believe to be now generally abandoned.

This is the most elegant method of any. No cement being employed, there is nothing on the slide except the sections that can stain, or appear as *dirt in the mount.* Tissues do not suffer from the drying, provided the material has been properly imbedded. Sections stick so fast by this method that they will stand staining on the slide; they will stand watery or other fluids for weeks, so long as they are not alkaline, as these may cause them to become detached. When successfully performed it is quite safe, provided that the sections are *of a suitable nature.* They must be such as to afford a sufficient continuous surface, everywhere in contact with the slide. Sections of parenchymatous organs stick well; sections of thin-walled tubular organs stick badly, often so badly that the method is really not safe for them at all. Sections of chitinous organs are very unsafe. The larger and thinner sections are, the better do they stick, and *vice versâ*. Sections from material that has been fixed in chromic or osmic mixtures adhere less well than sections from alcohol or sublimate material; so that for some sorts of material the method is certainly not safe. It has the disadvantage of being lengthy.

201. Mayer's **Albumen** (*Mitth. Zool. Stat. Neapel*, iv, 1883; *Internat. Monatschr. f. Anat.*, iv, 1887, p. 42).—White of egg, 50 c.c.; glycerin, 50 c.c.; salicylate of soda, 1 grm. Shake them well together, and filter into a clean bottle. The filtering may take days or a week, but the preparation does not spoil meanwhile.

I find it convenient to beat up the egg with a little water before adding the glycerin and filtering, the salicylate being dissolved in the water in the first instance.

A *very thin* layer of the mixture is spread on a slide with a fine brush and well rubbed in with the finger (previously well freed from grease by rubbing with alcohol). The sections are laid on it and pressed down lightly with a brush. The slide may then be warmed for some minutes on a water-bath, and the paraffin removed with a solvent.

It is *not necessary* to warm the slide at all; *the paraffin can be removed in the cold if desired* by putting the slide into toluol, xylol, or the like. But the slide *must*, in any case, be *treated with alcohol* after removal of the paraffin, in order to get rid of the glycerin, which will cause cloudiness if not perfectly removed.

The function of the glycerin is merely to keep the layer of albumen moist.

This method allows of the staining of sections on the slide with perfect safety, both with alcoholic and aqueous stains, provided they be *not alkaline*.

According to my experience, the albumen method is *absolutely safe*. It has the defect that certain plasma stains (not chromatin stains) colour the albumen very strongly, and cannot be removed from it.

It sometimes happens that the mixture after it has stood for some time becomes turbid, a change which has been attributed to the development of a microbe. I know of no means of preventing the mixture from going bad in this way, though I have found that it keeps better when freely exposed to the sun. It has been stated (VOSSELER, *Zeit. wiss. Mik.*, vii, 1891, p. 457) that after a time the mixture loses its adhesive properties, and should be thrown away (GRANDIS also [*Atti Accad. Lincei, Rend.* (4), vi, 1890, p. 138; *Arch. Ital. Biol.*, xiv, 1891, p. 412] states that the albumen of the mixture decomposes after a time). I find the liquid either first becomes milky, then altogether turbid, and at last coagulates, passing into a caseous state, or it may undergo a hyaline coagulation, drying up like amber. But up to the very last it does not in general lose its adhesive properties. I have, however, once found it to do so, after keeping for five or six years.

202. The Albumen and Water Method (HENNEGUY, *Journ. de l'Anat. et de la Physiol.*, 1891, p. 398).—A drop of water is spread on a slide painted with Mayer's white-of-egg mixture, the sections are arranged on it, the whole is warmed (*not* to the melting point of the paraffin) until the sections flatten out; the water is then evaporated off at a temperature of about 40° C., and as soon as it has sufficiently disappeared, which at that temperature will be in about ten to fifteen minutes, the paraffin is melted, and the slide further treated, described last §.

This is a most valuable method. It is quicker than the water method, and, for difficult material, safer.

See also the description of this method given by OHLMACHER (*Journ. Amer. Med. Ass.*, April, 1893), who has independently worked out the same process.

The so-called "Japanese" method, attributed to IKEDA by REINKE (*Zeit. wiss. Mik.*, xii, 1895, p. 21), is merely that of Henneguy.

203. OBREGIA'S **Method for Paraffin or Celloidin Sections** (*Neurologisches Centralb.*, ix, 1890, p. 295; GULLAND, *Journ. of Path.*, February, 1893).—Slides, or glass plates of any size, are coated with a solution made of—

Syrupy solution of powdered candy-sugar made with boiling distilled water	30 c.c.
95 per cent. alcohol	20 ,,
Transparent syrupy solution of pure dextrin made by boiling with distilled water	10 ,,

They are dried slowly for two or three days until the surface is just sticky to the moist finger. *Paraffin sections* are arranged and heated for a few minutes to a temperature slightly above the melting-point of the paraffin. The paraffin is removed by some solvent, such as xylol or naphtha, and this is in turn removed by absolute alcohol. The alcohol is poured off, and the sections are covered with solution of celloidin or with a solution of 3 per cent. of photoxylin or Schering's "Celloidinwolle" in a mixture of equal parts of ether and absolute alcohol. The plates are left to evaporate for ten minutes in a horizontal position, then brought into water, in which the sheet of celloidin with the sections soon becomes detached, and may be further treated as desired, *e.g.* as in Weigert's process, § 211. (It is well to divide the sheet of celloidin into ribbons by running the point of a knife down it as soon as evaporation has produced a very slight solidification, and the evaporation must not be artificially hastened.)

DIMMER (*Zeit. wiss. Mik.*, xvi, 1899, p. 44) coats the slides with a solution of about 16 parts of gelatin in 300 of warm water, and dries them (two days), and proceeds in other respects as above.

A good method for *large* sections, as the sheet of collodion can be easily manipulated as a single object in the subsequent operations of staining, etc., and any desired sections may be detached from it and mounted when required, the rest being preserved in the sheet till wanted. It is equally applicable to paraffin sections, to celloidin sections, and to sections of material that has not been imbedded at all.

For BLOCHMAN'S modification of Weigert's process, by means of which large sections can be preserved *unmounted*, see *Zeit. wiss. Mik.*, xiv, 1897, p. 189.

204. STRASSER'S Collodion Paper Method (*ibid.*, iii, 1886, p. 346).—

This is an extremely complicated modification of Weigert's method for celloidin sections, and is only adapted for use with STRASSER's automatic ribbon-microtome. See the original papers in *Zeit. wiss. Mik.*, iii, 1886, p. 346; vi, 1889, p. 154; vii, 1890, pp. 290 and 304; ix, 1892, p. 8; xii, 1895, p. 154; and xiv, 1897, p. 39; also (SCHOENEMANN) *ibid.*, xix, 1903, p. 333, and (STRASSER) *ibid.*, p. 337.

205. Other Methods for Paraffin Sections, now suppressed as being either faulty or superfluous, may be found described in *previous editions.*

Methods for Watery Sections.

206. FOL's **Gelatin** (FOL, *Lehrb.*, p. 132).—Four grammes of gelatin are dissolved in 20 c.c. of glacial acetic acid by heating on a water-bath and agitation. To 5 c.c. of the solution add 70 c.c. of 70 per cent alcohol and 1 to 2 c.c. of 5 per cent. aqueous solution of chrome-alum. Pour the mixture on to the slide and allow it to dry. In a few hours the gelatin passes into the insoluble state. It retains, however, the property of swelling and becoming somewhat sticky in presence of water. The slide may then be immersed in water containing the sections; these can be slid into their places, and the whole lifted out; the sections will be found to be fixed.

This method is specially intended for sections made under water, large celloidin sections amongst others.

Methods for Celloidin Sections.

207. The Albumen Method.—I find that celloidin sections may be mounted on Mayer's albumen, and have the celloidin removed, if desired, by putting them into ether-alcohol. Care must be taken to press them down very thoroughly on to the albumen.

So also JORDAN (*Zeit. wiss. Mik.*, xv, 1898, p. 54), who coagulates the albumen by heat, the sections being covered with a layer of tissue-paper and a second slide over it, to prevent them from drying through the heat. Similarly ARGUTINSKY, *ibid.* xvii, 1900, p. 37. For material cut by the oil of cedar method, § 183, JORDAN (*ibid.*, p. 192-194) employs either the albumen method or the albumen and water method, § 202, the sections (if I understand rightly) being brought on to the water whilst wet with the chloroform mixture; then after a few minutes' warming they are fixed, and the celloidin may be removed by alcohol and ether.

208. Summers' **Ether Method** (*Amer. Mon. Mic. Journ.*, 1887, p. 73).—Place the sections in 95 per cent. alcohol for a minute or two, arrange on the slide, and then pour over the sections sulphuric ether *vapour*, from a bottle partly full of liquid ether. The celloidin will immediately soften and become perfectly transparent. Place the slide in 80 per cent. alcohol, or even directly in 95 per cent. if desired. The sections, it is said, will be found to be firmly fixed, and may be stained if desired. I have not myself found this method safe.

Instead of pouring the ether vapour over the slide, it may, of course, be treated with ether vapour *in a preparation glass or similar arrangement*, which I think preferable.

Gage (*Proc. Amer. Soc. Mic.*, 1892, p. 82) advises that the slide be one that has been previously coated with a 0·5 per cent. solution of white of egg and dried; the collodion adheres much more strongly to an albuminised surface.

Auburtin (*Anat. Anz.*, xiii, 1897, p. 90) arranges on a clean slide, dehydrates the sections with blotting-paper and treatment with absolute alcohol, then drops on to them a mixture of alcohol and ether which dissolves out the celloidin from the sections, then allows the thin colloidion thus formed to evaporate into a thin sheet on the slide. Then 70 per cent. alcohol and other desired reagents.

209. Apáthy's **Oil of Bergamot Method** (*Mitth. Zool. Stat. Neapel*, 1887, p. 742; *Zeit. wiss. Mik.*, v, 1888, pp. 46 and 360).—Cut with a knife smeared with yellow vaselin and wetted with 95 per cent. alcohol. Float the sections, as cut, on bergamot oil (must be green, must mix perfectly with 90 per cent. alcohol, and must not smell of turpentine), or on carbolxyol (*Mikrotechnik*, p. 176). The sections *flatten themselves out* on the surface of the oil; before they sink each one is pushed by means of a needle into its place on a slip of tracing paper dipped into the oil. When the requisite number of sections have been arranged on the paper, you drain the paper, dry the under side of it with blotting-paper, turn it over, and gently press it down with blotting-paper on to a carefully dried slide. Remove the paper by rolling it up from one end. The sections remain adhering to the slide, and may have the remaining bergamot oil removed from them by means of a cigarette-paper. If they are already stained, nothing remains but to add balsam and a cover.

In the case of unstained or very small objects it is well to add a little alcoholic solution of safranin to the bergamot oil. The celloidin of the sections becomes coloured in it in a few seconds, and makes them readily visible. The colour disappears in a few days after mounting.

The process may be much simplified (Apáthy, *Mikrotechnik*, p. 127) by omitting the arrangement on the paper and transferring the sections direct from the bergamot oil to the slide, which (*ibid.*, p. 176) may have been previously collodionised and dried. The function of the bergamot oil is *to flatten out the sections.*

If the sections are to be stained, the slide after removal of the bergamot oil is exposed for a few minutes to the vapour of a mixture of ether and alcohol, then brought into 90 per cent. alcohol, and after a quarter of an hour therein may be stained in any fluid that contains 70 per cent. alcohol or more.

If it be desired to stain in a watery fluid, care must have been taken when arranging the sections to let the celloidin of each section overlap that of its neighbours at the edges, so that the ether vapour may fuse them all into one continuous plate. This will become detached from the slide in watery fluids, and may then be treated as a single section.

210. Apáthy's **Series-on-the-Knife Method** (*Zeit. wiss. Mik.*, vi, 1888, p. 168).—The knife is well smeared with yellow vaselin, rubbed evenly on with the finger, and is wetted with alcohol of 70 to 90 per cent. As fast as the sections are cut they are drawn with a needle or small brush to a dry part of the blade, and there arranged in rows, the celloidin of each section overlapping or at least touching that of its neighbours. The rows are the length of the cover-glass, and are arranged one under the other so as to form a square of the size of the cover-glass. When a series (or several series, if you like) has been thus completed, the sections are dried by laying blotting-paper on them (there is no risk of their becoming attached to it, as they are held down by the vaselin). The series is then painted over with some of the thinnest celloidin solution used for imbedding, is allowed to evaporate for five minutes in the air, and is then either wetted with 70 per cent. alcohol, and allowed to remain whilst cutting is proceeded with, or (if no more sections are to be cut, or if the knife is now full) the knife is removed and brought for half an hour into 70 per cent. alcohol. This hardens the celloidin around the sections into a continuous lamella, which can be easily detached by means of a scalpel, and stained, or further treated as desired. It is well to bring it at once on to a slide, moisten the edges of the celloidin plate with ether and alcohol mixture, so that it may not become detached, and bring the whole into the staining solution.

211. WEIGERT'S **Collodion Method** (*Zeit. wiss. Mik.*, 1885, p. 490).—Sections are cut wet with alcohol. Care should be taken not to have so much alcohol on the knife as to cause the sections to float. Prepare a slip of porous but tough paper (Weigert recommends "closet paper") of about twice the width of the sections. Soak it in alcohol, take it by both ends, stretch it slightly, and lower it on to the section that is on the knife. The section will adhere to the paper, and is taken up by moving the slip horizontally or slightly upwards, away from the edge of the knife. Take up the first section towards the end of the paper that you hold in your left hand, and let the remaining sections follow in order from left to right. After each section has been taken up, the slip is placed, whilst the next section is being cut, with the sections upwards on a moist surface prepared by arranging several layers of blotting-paper, covered with one layer of closet paper, in a plate, and saturating the whole with alcohol. When all the sections have been arranged on the slip, you pass to the next stage of the process, the collodionisation of the series.

This is done in two steps. The first of these consists in transporting the series on to a plate of glass prepared with collodion. The plate is prepared beforehand by pouring on to it collodion and causing it to spread out into a thin layer, as photographers do, and allowing it to dry. (A number of the plates may be prepared and kept indefinitely in stock ; microscope slides will do for series of small sections.) Take one of these plates; lay the slip of paper with the sections on the plate, the sections downwards; press it down gently and evenly, and the sections will adhere to the collodion; then carefully remove the paper. (Do not place more than one or at most two lines of sections on the same plate, for those first placed run the risk of becoming dry whilst you are placing the others.) This finishes the first stage of the collodionising process.

Now remove with blotting-paper any excess of alcohol that may remain on or around the sections, pour collodion over them, and get it to spread in an even layer. As soon as this layer is dry at the surface you may write any necessary indications on it with a small brush charged with methylen blue (the colour will remain fast throughout all subsequent manipulations).

The plate may now be either put away till wanted in 80 per cent. alcohol, or may be brought into a staining fluid. The watery fluid causes the double sheet of collodion to become detached from the glass, holding the sections fast between its folds. It is then easy to stain, wash, dehydrate, and mount in the usual way, merely taking care not to use alcohol of more than 90 to 96 per cent. for dehydration. Weigert recommends for clearing the mixture of xylol and carbolic acid (§ 179).

The series should be cut into the desired lengths for mounting whilst in the alcohol. It is perhaps safer to lay them out for cutting on a strip of closet paper saturated with alcohol.

A good method for *large* and *thick* sections that do not require flattening, not for series of small thin ones.

It is suggested by STRASSER that gummed paper might be an improvement on the glass plates used in this process—especially for very large sections. See hereon the papers quoted § 204.

The modification of Weigert's method proposed by WINTERSTEINER (*Zeit. wiss. Mik.*, x, 1893, p. 316) consists in suppressing the alignment of the sections on the strip of paper, and slipping them direct from the knife on to the prepared glass.

For BLOCHMAN'S modification see § 203.

212. Obregia's Method.—Slides are prepared as directed (§ 203), the sections are taken up in order on a strip of paper (glossed tissue paper, *satinisirtes Seidenpapier,* the sections to be on the glossed side) as in WEIGERT'S method, and laid down on the glass in the same way, and then covered with the celloidin or photoxylin solution and evaporated as described.

For DIMMER'S modification see § 203.

213. GIACOMINI'S collodion-gelatin process for large sections, see *Gazzetta delle Cliniche*, November, 1885, *Zeit. wiss. Mik.*, 1885, p. 531, or the first edition of the *Traité* of LEE et HENNEGUY, p. 392.

CHAPTER XI.

STAINING.

214. The Molecular Processes involved in Staining.—The question whether the phenomena of staining and of industrial dyeing are chiefly of a chemical order, as held by some, or chiefly of a physical order, as held by others, is outside the province of this book. See (besides works on chemistry, amongst which may be mentioned BENEDIKT and KNECHT, *The Chemistry of the Coal-tar Colours*, London, 1889) FISCHER'S *Fixirung, Färbung und Bau des Protoplasmus*, Jena, G. Fischer, 1899; PAPPENHEIM'S *Grundriss der Farbchemie*, Berlin, A. Hirschwald, 1901; and the articles in *Encycl. mik. Technik*, 1903.

215. Histological Staining: Specific, Nuclear, and Plasmatic.—Stains are either general or special (otherwise called Specific, or Selective, or Elective). A general stain is one that takes effect on all the elements of a preparation. A special, specific, selective, or elective stain is one that takes effect only on some of them, certain elements being made prominent by being coloured, the rest either remaining colourless or being coloured with a different intensity or in a different tone. To obtain this *differentiation* is the chief object for which colouring reagents are employed in microscopic anatomy.

Two chief kinds of this selection may be distinguished—*histological* selection and *cytological* selection. In the former an entire tissue or group of tissue elements is prominently stained, the elements of other sorts present in the preparation remaining colourless or being at all events differently stained, as in a successful impregnation of nerve-endings by means of gold chloride. This is the kind of stain that is generally meant by a *specific* stain. In the latter the stain seizes on one of the constituent elements of cells in general, for instance either on the chromatin of the nucleus, or on one or other of the elements that go to make up the cytoplasm.

Stains that thus exhibit a selective affinity for the substance of nuclei—*nuclear* or *chromatin* stains—form a class of stains of peculiar importance for the embryologist or zootomist. What the zootomist or embryologist wants, in the great majority of cases, is not so much to differentiate the intimate structures of cells by means of a colour reaction, in order to study them for their own sakes, as the cytologist does, as merely to have the nuclei of tissues marked out by staining in the midst of the unstained material in such a way that they may form landmarks to catch the eye, which is then able to follow out with ease the contours and relations of the elements to which the nuclei belong; the extranuclear parts of these elements being expressly left unstained (or only lightly tinged) in order that as little light as possible may be absorbed in passing through the preparation—a procedure which is found in practice to be very efficient for general work.

To these must be added another group of stains of the greatest importance to the cytologist and histologist, the *plasmatic stains* or *plasma stains.* These take effect especially on elements of cells and tissues other than the chromatin—for instance, on the reticulum of cytoplasm, or on its granules, or on polar corpuscles, etc., or on the formed material of tissues—the chromatin being left as far as possible unstained, in order that it may be counterstained in another colour by means of one of the above-mentioned chromatin stains.

In this book, therefore, stains are looked upon as being (1) General stains; (2) Selective stains; the latter group being subdivided into (*a*) Nuclear, (*b*) Plasmatic, (*c*) Histologically Selective, or Specific.

216. Dyes: Basic, Acid, and Neutral.—The colouring matters, or dyes, employed either in industrial dyeing or in histological staining are almost always *salts.* They are known as "*basic,*" "*acid,*" or "*neutral*" dyes. By a "basic" dye is meant a compound in which a so-called "colour base" (or molecular group to which the compound owes its colouring properties) is combined with a non-colouring acid. For instance, fuchsin or magenta is a basic dye. It is the hydrochloride of rosanilin, and its colouring properties are due to the rosanilin which exists as a base in the compound, and not to the hydrochloric acid of the compound. By an "acid" dye is meant a compound in which a so-called "colour-acid" is combined with a non-colouring base. The dye known as acid fuchsin or acid magenta (Säurefuchsin) is an "acid" colour. It is the soda salt of di- or tri-sulphoconjugated rosanilin, that is of rosanilin di- or tri-sulphonic acid, and its colouring properties are due to the rosanilin which exists as an acid in the compound, and not to the soda. Or to take a simpler case, picrate of ammonia is an "acid" colour, and its colouring properties are evidently due to the picric acid in it, and not to the ammonia.

It is important to keep clearly in mind that in speaking of dyes the terms "acid" or "basic" refer to the characters of the colour acids or colour bases, and not to those of the salts. An "acid" dye may have a neutral or alkaline reaction (*e. g.* picrate of ammonia), and *vice versâ.*

Basic dyes are generally easily soluble in alcohol, less easily in water; whilst the contrary is the case for acid dyes. The free colour bases or colour-acids are generally less soluble in water than their salts, for which reason they are so little used that they are hardly to be found in commerce. It follows that such histological formulæ as depend on setting free a colour-acid from its salt (*e. g.* as by precipitating it from eosin by means of alum, as advised by Ranvier and Wissotzky) are irrational. Colour-bases or colour-acids may themselves be colourless.

The stain given by acid dyes is fast to acids, and may be intensified by them; whilst basic dyes are washed out by acids, but intensified by alkalies.

"Neutral" dyes are compounds of a colour-base with a colour-acid. They are seldom or never prepared industrially, the only example that I can find mentioned in BENEDIKT and KNECHT'S *Chemistry of the Coal-tar Colours* being artificial indigo. They are prepared for histological purposes by mixing the aqueous solutions of a basic and an acid dye. For instance, by mixing the acid picrate of ammonia with the basic hydrochloride of rosanilin, you can bring about the formation of sal ammoniac and picrate of rosanilin, which is a "neutral" colouring matter. They are generally insoluble in pure water, and hence precipitate when the mixture is made, but may be got to redissolve by adding an excess of the acid colour, or of the basic, and are always soluble in alcohol. They can also, as is often done, be formed in the tissues themselves by staining first with an acid dye, and then bringing the preparation, without first washing out, into a basic dye.

See further as to the "neutral" colours, ROSIN, "Ueber eine neue Gruppe der Anilinfarbstoffen," in *Berliner klin. Wochenschr.*, xii, 1898, p. 251; *Zeit. f. wiss. Mik.*, xvi, 2, 1899, p. 223; *Journ. Roy. Mic. Soc.*, 1899, p. 547; *Encycl. mik. Technik*, 1903, p. 1028; PAPPENHEIM, *op. cit. supra*; HEIDENHAIN, *Anat. Anz.*, xx, 1901, p. 36.

217. The Chromatophily of Tissue-Elements.—The elements of tissues are distinguished as "basophilous," "acidophilous," and "neutrophilous" according as they seem to show a natural affinity for basic, acid, or neutral colouring matters respectively. According to a generalisation due to EHRLICH (*Zeit. klin. Med.*, 1, 1880, p. 555; REICHERT AND DU-BOIS REYMOND'S *Arch. Anat. Phys., Phys. Abth.*, 1879, p. 571), the basic colours are in general chromatin stains—that is, they have a special affinity for the element of nuclei known as chromatin, so that they are mostly sharp nuclear stains, and chromatin is *basophilous*. The acid colours, on the other hand, are, according to him, in general plasma stains—that is, they have a special affinity for cytoplasm and intercellular substances, which are therefore *acidophilous*. The neutral colours exhibit special affinities for certain cell-contents and the elements affected by these are said to be *neutrophilous*.

I think that that is a generalisation which requires some explanation and qualification. In practical histology we have to take account not only of the affinities for dyes of cellular elements in a physically and chemically unaltered state, but of the alterations in these affinities brought about by the action of fixatives and mordants. Now most fixing agents either diminish or increase the chromatophily of tissues; so also do all mordants, some of which may even invert the natural chromatophily of tissues (see §§ 28, 218, 220). Then, too, we have to take account also of the resistance of the stain to the liquids employed for washing, for dehydration, for clearing; in short, we have to take into account the way in which the dye behaves when employed as a *regressive* stain (§ 222). This is of peculiar importance in the case of the coal-tar colours, seeing that they are largely used for the *regressive* staining of sections destined to be dehydrated by alcohol and mounted in balsam. Now Ehrlich's experiments take no account of these conditions. (He worked with "cover-glass preparations" of isolated cells, such as blood and lymph cells, and was thus able to avoid the prolonged washing necessary for most sections, and to suppress altogether the dehydration by alcohol, his cover-glass preparations being simply dried after staining in a stove.) In consequence, his chemical categories of *basic colours* and *acid colours* fail to correspond always *in practice* to the technical categories of *chromatin stains* and *plasma stains.*

For instance, orange is an acid colour; but used as a regressive stain I find it will give a very sharp stain of chromatin: it cannot, therefore, be classed as a mere plasma stain, though it is also a very good plasma stain. Säurefuchsin is a very acid colour. It behaves in general as a decided plasma stain. But used as a regressive stain it sometimes, under conditions which I am not able to specify, gives a very vigorous stain of chromatin. Safranin is a basic colour, but by the use of appropriate mordants it can be made to behave as a plasma stain. Methylen blue is a basic colour. But, as is well known, when employed according to the method worked out by Ehrlich for the so-called *intra-vitam* staining of nerves, it affords a stain that is essentially plasmatic, such staining of nuclei as may occur in this process being an accidental epiphenomenon. Nigrosin is, according to Ehrlich, an acid colour, and should therefore be essentially a plasma stain. Yet I find that, used as a regressive stain in the same way as safranin, it gives a vigorous chromatin stain, cytoplasm being only faintly coloured. Bordeaux is an acid colour, but it stains chromatin as well as cytoplasm. Further, both carminic acid and hæmatein are acid dyes, but combined with the mordant, alum (as in alum-carmine or alum-hæmatoxylin), they give nuclear stains. Indeed, it is not too much to assert that there is hardly any colour, either basic or acid, that may not be made to afford either a chromatin stain or a plasma stain, according to the way in which it is employed.

I do not in the least doubt that Ehrlich's generalisation is theoretically exact. It is no doubt true that nuclein, being an acid body, has a natural affinity for colour-bases, and that cytoplasm, being generally basic, has a natural affinity for colour-acids; and it is no doubt roughly true in practice that basic dyes are in general chromatin stains and acid dyes plasma stains; but the rule does not hold under *all conditions*. There is no absolute chromatophily of tissue elements.

218. Substantive and Adjective Staining; Mordants.—In the industry of dyeing, colouring matters are divided into two classes, according to their behaviour with respect to the material to be dyed. Certain dyes are absorbed directly from their solutions by the material immersed therein, and combine with it directly. In this case the material is said to be *substantively* dyed, and the colouring matter is called a *substantive* colouring matter.

Other dyes do not combine directly with the material to be acted on, but this material must first be charged with some substance known as a *mordant* (generally a metallic salt or hydrate) before it will combine with the colouring matter. These are known as *adjective* colouring matters.*

Mordants are bodies which have the property of combining on the one hand with the elements of tissues and on the other with the colouring principle of the dyes used, forming with the latter insoluble coloured compounds (known as Lakes), which are retained in the tissues. It follows that basic dyes require mordants of an acid character and acid dyes mordants of a basic character. The mordant may be introduced into the tissues either before the dye or at the same time with it.

It may seem at first sight that the majority of histological stains are obtained by substantive staining of the tissues. But on reflection it will be seen that many of the histological stains that are obtained without intentional mordanting of the tissues should yet in strictness be attributed to the class of adjective stains. For whenever there is reason to suppose that the stain obtained results from a combination of the colouring matter with some metallic salt or hydrate that is not a constituent of the living tissue, but has been brought into it by the fixing or hardening reagents, it must be admitted that these reagents play the part of mordants though only intentionally employed for another purpose. This would appear to be the case with the stains, or some of them, obtained after fixation with corrosive sublimate, picric acid, salts of iron, of platinum, of palladium, of uranium, and, for certain tissue elements and certain colours, chromium. And further, the mordanting substance may not only be present unintentionally in the fixing or hardening agents, it may be present unintentionally, or with imperfect realisation of its import, in the staining solutions themselves. Such is undoubtedly the part played by alum in most of the stains in which it figures as an ingredient. Iodine also plays in some staining processes a part which seems only explicable on the supposition that it acts as a mordant. And in some processes an acid dye is made to act as a mordant for a subsequently employed basic dye.

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* For an excellent popular exposition of this subject see BENEDIKT and KNECHT's *Chemistry of the Coal-tar Colours* (George Bell and Sons).

In some staining processes, however, mordants are *intentionally* resorted to in order to fix the stain. Mordanting has long been intentionally employed in some hæmatein staining processes, such as the iron-alum process of BENDA and M. HEIDENHAIN. More lately it has been resorted to for staining with tar colours.

By combining with the elements of tissues, mordants confer on them an artificial chromatophily. This may take place to so great an extent that the original affinities of the tissues for dyes are not only masked but may be seemingly altered into their opposites, thus producing an "inversion" of their chromatophily. Thus by means of appropriate acid mordants certain basic anilins, which by the usual methods of regressive staining are pure chromatin stains, may be made to afford a pure plasma stain—one not affecting chromatin at all, thus giving an "inversion" of the usual stain.

It would seem that the nuclear stains obtained by carmine and hæmatoxylin should in strictness be classed as inversion stains. The colouring principle of carmine, carminic acid, is an acid body, and consequently a plasma stain. But in the form of carmine it is combined with the basic oxide alumina, which by mordanting the acid body nuclein confers on it a basic character and renders it acidophilous, thus producing an inversion of the natural staining affinity. So also with the acid dye hæmatein. So that we see that whilst basic dyes substantively employed are nuclear stains and acid dyes substantively employed are plasma stains, yet basic colours with acid mordants may give a plasma stain and acid colours with basic mordants may give a nuclear stain.

219. Metachromasy.—Adjective stains are of the colour of the lake formed by the mordant and the colouring principle of the dye. Substantive stains are for the most part of the colour of the solution of the dye employed. But this is not always the case. There is a very small group of dyes, mostly basic tar-colours, which have the property of staining different elements of tissues in different colours, one of these being the same as that of the solution of the dye, and the other, or metachromatic colour, being the same as that of the free colour-base of the dye. For instance, the red dye, safranin, colours nuclei red, but mucin and the ground-substance of cartilage orange. The blue dye, methyl violet, stains normally blue; but amyloid matter, Mastzellen, mucin, and cartilage, are stained red by it. Similarly with thionin, dahlia, gentian violet, toluidin blue, etc.

In these cases the effect appears to be due to chemical reaction. But in other cases a similar effect has been shown to be due to the presence of impurities in the dyes; so with iodine green and methyl green, which mostly contain an admixture of methyl violet.

It has been held by some that metachromasy is an optical phenomenon.

220. The Preparation of Tissues for Staining.—It is *generally* found that precise stains can only be obtained with carefully *fixed* (*i. e.* hardened) tissues. Dead, but not artificially hardened tissues stain indeed, but not generally in a precise manner. Living tissue elements in general do not stain at all, but resist the action of colouring reagents till they are killed by them (see, however, next section).

It appears probable, as was first pointed out, I believe, by Paul Mayer, that most of the histological stains obtained with fixed tissues are brought about in one of two ways. Either they result from the combination of the colouring agent with certain organic or inorganic salts,—phosphates, for instance, that existed in the tissue elements during life and were thrown down *in situ* by the fixing or hardening agent employed, as seems to happen when such a fixing agent as alcohol is employed. Or they result from the combination of the colouring agent with certain compounds that did not pre-exist in the tissues, but were formed by the combination of the constituents of the tissues with the chemical elements brought to them by the fixing agent, as seems to happen when such a fixing agent as chromic acid is employed —the compounds in question being probably chiefly metal albuminates. It follows that *correct fixation* and careful *washing out* are essential to the production of good stains. See §§ 27, 28.

Long preservation of tissues in alcohol is generally unfavourable to staining.

221. Staining "intra vitam."—Some few substances (which are almost always *basic* dyes) possess the property of staining —or rather, tingeing—living cells without greatly impairing their vitality. Such are—in very dilute solutions—cyanin (or quinoleïn), methylen blue, Bismarck brown, anilin black, Congo red, neutral red, Nile blue, Janus green, and, under certain conditions, dahlia and eosin, gentian violet, with perhaps methyl violet, and some others whose action is not yet sufficiently established by experiment.

These reagents are best employed in a state of considerable dilution, and in neutral or feebly alkaline solution—acids being of course toxic to cells. Thus employed, they will be found to tinge with colour the cytoplasm of certain cells during life ; never, so far as I can see, nuclear chromatin during life ;—if this stain, it is a sign that death has set in. The stain is sometimes diffused throughout the general substance of the cytoplasm, sometimes limited to certain granules in it.

It has been asserted by some observers that the nucleus may be stained during the life of the cell by means of Bismarck brown, Congo red, methylen blue, neutral red, Nile blue, and safranin. But it is by no means clear from the statements of these writers that the coloration observed by them is localised *in the chromatin* of the nucleus. It would rather appear to be a diffuse coloration brought about by mechanical retention of the dye in the nucleus—which is a very different thing from a true nuclear stain. And in some of the cases reported it is by no means certain that the coloured nuclei were really in the living state. See hereon the article by Fischel ("Färbungen, intravitale") in *Encycl. mik. Technik.*

I have myself made a considerable number of observations on the subject of *intra-vitam* staining, and have come to the same conclusion as Galeotti (*Zeit. wiss. Mik.*, xi, 1894, p. 172), and many recent writers, namely that most of the so-called "intra-vitam" stains are either not true stains or that the stained substances are not really living. The diffuse coloration above mentioned appears always, if the cell that shows it has remained in a state of unimpaired vitality, to be due to simple absorption or imbibition of the colouring matter by the cell, not to a molecular combination of the colouring matter with any of the constituents of the cells. If a cell thus coloured be transported into a medium free from the colouring matter it will give up unchanged the colour it had imbibed, which seems to be a sufficient proof that the colouring matter had not entered into any molecular combination with the elements of the cell, but was simply loosely held in a mechanical way in the interstices of its substance. If, on the other hand, there has been produced the above-mentioned coloration of certain granules or other cell-contents, it is possible that this may be a true stain in the sense of being such a combination as is formed in *fixed* material when stained. It may be so, but it certainly is not always so, as may sometimes be proved with the greatest ease by putting the cell into a colourless medium and observing the supposed stain disappear. And in cases in which this does not happen, in which, therefore, a more or less fast stain has been obtained, it is generally found that the stain is limited to cell-contents that do not appear to form an integral part of the living texture of the cell; the cell itself may be living, but they are not. These granules or other cell-contents may be granules formed of substances that have been absorbed by the cell from without—food-granules; or they may be katabolic products, consisting of matter that is no longer alive and is destined to be shortly expelled from the cell; or they may be elements that form indeed an integral part of the living texture of the cell, but have been injuriously affected by the colouring matter, and for that or some other reason are in a state of diminished vitality,—they are parts of the cell that are being killed by the colouring reagent or that have been totally killed by it, whilst the rest survives. It is difficult to show that they consist of matter that is fully and perfectly alive. I am inclined to think, indeed, that the so-called vital or *intra-vitam* stains may be found to furnish us with the means of distinguishing the living constituents of a cell from the non-living ones, and even of recognising amongst the living ones those that possess only a relatively low or impaired degree of vitality. See on this point (as on others connected with the theory of staining) the work of Fischer, quoted § 214.

Fischel (*Anat. Hefte*, xvi, 1901, p. 417, and *op. cit. supra*) concludes in favour of the vitality of *certain* of the stained granules.

Apart, however, from the question whether the elements stained by the so-called "vital" stains are truly living or not, it must be conceded that this mode of treating living cells has frequently a considerable measure of practical utility. It often enables us to map out physiological or morphological tracts that would otherwise be unrecognisable or less readily recognisable in the living state, and aids in the study of cell granules and processes of secretion and the like. According to my experience, methylen blue is the most generally useful of these stains. It has (with Bismarck brown, Congo red, and neutral red) the valuable point that it is sufficiently soluble in saline solutions, and may therefore be employed with marine organisms by simply adding it to sea-water. The others are not thus soluble to a practical extent, but I find that gentian and dahlia become so if a trace of chloral hydrate—0·25 per cent. is ample enough—be added to the saline solution. Any of these reagents may be rubbed up with serum, or other "indifferent" liquid.

Methylen blue may be fixed in the tissues, and permanent preparations made, by one or other of the methods described in Chap. XVI. Bismarck brown stains may be fixed with 0·2 per cent. chromic acid or with sublimate solution (Mayer), or 1 per cent. osmic acid (Loisel, *Journ. de l'Anat. et de la Phys.*, 1898, No. 2, p. 212—a work that contains a good deal of information on the subject of *intra-vitam* stains), and the preparations may be stained with safranin, care being taken not to expose them too long to the action of alcohol.

For the study of cell-granules neutral red is perhaps the best.

222. The Practice of Staining.—Colouring matters possessing so great an affinity for certain elements of tissues that they may be left to produce the desired electivity of stain without any special manipulation on the part of the operator are unfortunately rare. In practice, selective staining is arrived at in two ways. In the one, which is called the *progressive* method, you make use of a colouring reagent that stains the elements desired to be selected more quickly than the elements you wish to have unstained; and you stop the process and fix the colour at the moment when the former are just sufficiently stained, and the latter not affected to an injurious extent, or not affected at all, by the colour. This is what happens, for instance, when you stain the nuclei of a preparation by treatment with very dilute alum hæmatoxylin: you get, at a certain moment, a fairly pure nuclear stain; but if you were to prolong the treatment, the extra-nuclear elements would take up the colour, and the selectivity of the stain would be lost. This is in general the method employed for the colouring of specimens *in bulk*,—a procedure which is not possible with most of the regressive stains. It is the old method of carmine and hæmatoxylin staining.

The second, or *regressive* method, is the method of overstaining followed by partial decoloration. You begin by staining all the elements of your preparation indiscriminately, and you then wash out the colour from all the elements except those which you desire to have stained, these retaining the colour more obstinately than the others in virtue of their chemical or physical constitution. This is what happens, for instance, when you stain a section of one deep red in all its elements with safranin, and then, treating it for a few seconds with alcohol, extract the colour from all but the chromatin and nucleoli of the nuclei. This method is in general applicable *only to sections*, and not to staining objects *in bulk* (the case of borax carmine, with a few others, is an exception). It is a method, however, of very wide applicability, and gives, perhaps, the most brilliant results that have hitherto been attained. It frequently enables us to obtain a powerful stain of certain elements that would not be sufficiently brought out by the progressive method.

Tissues are stained either in *bulk* or in *sections*. For accurate work, such as is necessary in cytology and frequently in histology, it is greatly preferable, sometimes even necessary, to stain the sections, as by this means only is accurate control of the staining process under the microscope possible. And the most accurate results are only obtainable with cells and nuclei that have been *incised*.

Staining solutions are mostly made with either *water* or *alcohol* as a menstruum. Water is generally preferable so far as the quality of the stain is concerned ; but alcohol is frequently indicated, both on account of its greater power of penetration and as being less injurious to tissues. It is a good rule not to let staining baths contain more than fifty per cent. of alcohol.

Better results (as regards the quality of the stain, not as regards the preservation of the tissues) are generally obtained by *prolonged* staining in very dilute solutions, rather than by a *short* bath in a strong one. This applies chiefly to the progressive method, but is also applicable to the regressive method, which does not give the most accurate differentiations with tissues overcharged with colour.

223. Choice of a Stain.—The following may be recommended to the *beginner* for general work :—*For sections,* MAYER'S *hæmalum ;* or, for chromosmium objects more especially, BENDA'S or HEIDENHAIN'S iron hæmatoxylin.

For staining *in toto* Grenacher's alcoholic *borax-carmine,* or Mayer's *carmalum,* or hæmalum, unless the object be so impermeable as to require a very highly alcoholised stain, in which case take Mayer's *paracarmine,* or for chromic acid objects Mayer's *hæmacalcium.*

For *fresh tissues* or small entire objects, *methyl green,* if it is not important to have permanent preparation ; if it is, take *carmalum* or *alum-carmine* (but both of these may give precipitates with marine animals).

224. Staining Reagents and Chemicals.—You are not likely to succeed in staining, especially in staining with coal-tar colours, unless you see to it that you are working with chemicals of the proper quality. You *cannot* ensure this by going to a generally trustworthy house for chemical products —at all events, not in the case of coal-tar colours. It is not sufficient that these should be what they are commercially described to be; they may be pure, and yet not give good stains. They must (in the case of coal-tar colours, at all events) be the identical products used in their work by the authors who have described and recommended them (see the note on the numerous safranins in the market, *s. v. Safranin*). I therefore advise the reader to get his reagents—at all events his dyes—from the well-known chemists GRÜBLER & HOLLBORN or MÜNDER. They may be ordered from the price list, or by quoting the numbers of the formulæ in this work. The address is: Herrn Dr. G. GRÜBLER & HOLLBORN, Chemiker, Baiersche Strasse 63, Leipzig. Their preparations can be obtained in London from Mr. CHARLES BAKER, 244, High Holborn, W.C., who is also agent for the microscopes and apparatus of Zeiss, also for the microtomes of Jung, Becker, and others, and the bacteriological apparatus of F. and M. Lautenschlaeger, etc.

Münder's address is: Herrn Dr. G. MÜNDER, Mikroskopisch-chemisches Institut, Göttingen.

CHAPTER XII.

CARMINE AND COCHINEAL STAINS.

225. Carmine.—Carmine is by no means merely carminic acid with at most certain impurities. According to the analysis of LIEBERMANN (*Ber. d. Chem. Ges.*, Jahrg. 18, 1886, pp. 1969—1975) it is a *very peculiar alumina-lime-protein compound of carminic acid,* a true chemical compound from which at all events *aluminium* and *calcium* can no more be absent than sodium from salt. Analysis gave him about 17 per cent. of water, 20 per cent. nitrogenous matters, 56 per cent. carminic acid, at least 3 per cent. alumina, and 3 per cent. lime, together with a small proportion of magnesia, potash, soda, phosphoric acid, and a trace of wax. It results from the researches of PAUL MAYER (*Mitth. Zool. Stat. Neapel,* x, 1892, p. 480) that in the processes of histological staining (*not* of industrial dyeing) the active factors of the compound are, besides the carminic acid, always the *alumina,* and in some cases the lime. *The other bases are inactive;* the nitrogenous matters, so far as they have any influence at all, are an obstacle, as it is they that give rise to the well-known putrefaction of the solutions.

This being so, it seems logical to admit that *carminic acid, instead of carmine, should be taken as the basis of staining solutions.* This had already been proposed by DIMMOCK (*Amer. Natural.,* xviii, 1884, pp. 324–7). But Dimmock's proposals were not very successful, for the reason that he had omitted from his solutions the essential element, the alumina. He stained, for instance, with pure alcoholic solution of carminic acid, or of carminate of ammonia. Such solutions stain, but stain weakly, and diffusely. So that in practice it is necessary either to take carmine as the basis of the solutions, or to combine the carminic acid either with *aluminium* according to the processes worked out by MAYER (next §), or with some other mordants. Methods employing *iron* as a mordant are given §§ 236—240. All these processes are explicable by the consideration that carminic acid is an "acid" dye (§ 216), giving, therefore, substantively a plasma stain; so that a mordant must be added to make it give a nuclear stain (§ 218).

226. Carminic Acid occurs as a purple-brown mass, easily soluble both in water and in alcohol. It is (according to NIETZKI, *Chemie der organischen Farbstoffe*, Berlin, 1889, pp. 231—234) a weak (LIEBERMANN says a strong) dibasic acid, which forms soluble salts with the alkaline metals, insoluble violet-coloured ones with the earthy and heavy metals.

The alumina salt (carminate of alumina) has the remarkable property of being soluble not only in acids and acid salts, such as alum, but also in alkalies and alkaline salts, such as borax, provided that only water or weak alcohol be employed as the menstruum. It may be obtained by precipitating a solution of carminic acid or of carminate of ammonia by means of acetate of alumina. It is also precipitated from the above-named solutions by chloride of aluminium, but only in part; whilst if alum be taken no precipitate is produced, the carminate of alumina remaining in solution, and forming the staining fluid given below under the name of **Carmalum.**

When chloride of aluminium is taken, a precipitate is formed, as stated above. But this precipitate will redissolve if more chloride of aluminium be cautiously added. This gives the staining fluid described in § 230, which may be convenient in cases in which it is not desirable to work with a fluid containing alum.

Both of these solutions stain in a violet tone, something like alum-carmine. A redder tone may be obtained by adding calcium chloride to the carmalum solution. But this is not advisable, for calcium chloride added to carmalum precipitates the solution with formation of gypsum. Of course, this does not occur with the aluminium chloride solution; but for other reasons the addition does not give satisfactory results with the chloride of aluminium solution mentioned above. But it does give good results when combined with an *alcoholic* chloride of aluminium solution, and thus solves at once the problem of obtaining a *red* stain and an *alcoholic* staining fluid. This is described below under the name of **Paracarmine.**

227. Cochineal.—According to Mayer (*Mitth. Zool. Stat. Neapel,* x, 1892, p. 496), the active principle of extract or tincture of cochineal (as used in histology) is not free carminic acid, but carminic acid chemically combined with a base which is not lime, but some alkali. The pure aqueous extract contains only traces of lime, the alcoholic none at all. The watery extract made with *alum,* or cochineal-alum carmine (§ 232), owes its staining power to the formation of carminate of alumina (last §). The tincture made with *pure alcohol,* on the other hand, contains only the above-mentioned carminate of some alkali. This carminate *alone* stains weakly and diffusely (like carminic acid alone). But if in the tissues treated with it it meet with lime salts, alumina or magnesia salts, or even metallic salts capable of combining with it and forming insoluble coloured precipitates in the tissues, then a strong and selective stain may result. As a matter of fact, the simple cochineal tincture of Mayer given in § 252 does give splendid results with certain objects (*i.e.* such as contain the salts in question). But it is unfortunately equally certain that such objects are rather rare than otherwise, and that with the majority of objects the stain is a very poor one.

But if the necessary salts be added to the tincture itself, there results a solution containing the necessary elements for affording a strong and selective stain with all classes of objects. Hence Mayer's new formula, § 253.

228. General Remarks.—Carmine stains are chiefly used for staining *entire objects,* or tissues in bulk. In most cases this can be done more satisfactorily by means of carmine than by means of any other known agent. For most hæmateïn solutions have a disastrous tendency to overstain; and the tar-colours are generally inapplicable to staining in bulk.

Grenacher's alcoholic borax-carmine may be recommended to the beginner as being the easiest of these stains to work with. Carmalum, or one of the alum-carmines, is also an easy and safe reagent.

Overstains may in all cases be washed out with weak HCl (*e. g.* 0·1 per cent.). Alum-solution will often suffice, or, according to HENNEGUY (*Journ. de l'Anat. et de la Physiol.*, xxvii, 1891, p. 400), permanganate of potash. All carmine stains, with the exception of aceto-carmine, are permanent in balsam. None of the acid stains, nor any of Grenacher's fluids, should be used with calcareous structures that it is wished to preserve, unless they be taken in a state of extreme dilution.

A. AQUEOUS CARMINE STAINS.

a. Acid.

229. MAYER'S **Carmalum** (*Mitth. Zool. Stat. Neapel*, x, 1892, p. 489).—Carminic acid, 1 grm.; alum, 10 grms.; distilled water, 200 c.c. Dissolve with heat (if necessary: I have been able to make my solutions in the cold). Decant or filter. Add some antiseptic, either a few crystals of thymol, or 0·1 per cent. salicylic acid, or 0·5 per cent. salicylate of soda. The solution will then keep. A clearish red fluid with a violet tinge. It stains well in bulk even osmium objects. If washed out with distilled water only, the plasma will remain somewhat stained. If this be not desired, wash out carefully with alum solution, or, in difficult cases with weak acid, followed in either case with water. The general effect is that of an alum-carmine stain. A notable difference between the two is that carmalum stains well in bulk, which alum-carmine is not very suitable for when used in the ordinary way; but see § 233.

A weaker solution may be made by taking from three to five times as much alum and five times as much water, and dissolving in the cold, which may be convenient. This is a very close equivalent of alum-carmine, giving, however, a somewhat redder stain. I find this solution very weak for ordinary work.

With either solution the objects to be stained should *not* have an *alkaline reaction.* The other properties of these solutions are very similar to those of alum-carmine.

RAWITZ (*Anat. Anz.*, xv, 1899, p. 438) takes 2 grms. carminic acid, 20 grms. ammonia-alum, 150 c.c. water, and 150 c.c. glycerin. A strongly staining solution, which is said to keep well. He recommends it only for sections.

230. Mayer's Aqueous Aluminium-Chloride-Solution (*Mitth. Zool. Stat. Neapel*, x, 1892, p. 490).—Carminic acid, 1 grm.; chloride of aluminium, 3 grms.; water, 200 c.c. Add an antiseptic, as for carmalum.

Use as carmalum. The stain is of a blue-violet colour, very powerful, and elective, but not so purely nuclear as carmalum. It is recommended only as a substitute for carmalum in cases in which the latter is counter-indicated on account of the presence of alum or the like.

231. Alum-carmine (Grenacher, *Arch. mik. Anat.*, xvi, 1879, p. 465).—An aqueous solution (of 1 to 5 per cent. strength, or any other strength that may be preferred) of common or ammonia alum is boiled for ten or twenty minutes with ½ to 1 per cent. of powdered carmine. (It is perhaps the safer plan to take the alum solution highly concentrated in the first instance, and after boiling the carmine in it dilute to the desired strength.) When cool, filter.

This stain must be avoided in the case of calcareous structures that it is wished to preserve.

Tizzoni (*Bull. Sc. Med. Bologna*, 1884, p. 259), Pisenti (*Gazz. degli Ospetali*, No. 24; *Zeit. wiss. Mik.*, ii, 1885, p. 378), and Grieb (*Mem. Soc. Ital. Sci.*, t. vi, No. 9, 1887; *Zeit. wiss. Mik.*, vii, 1, 1890, p. 47) have given modifications of Grenacher's formula which do not appear to me rational.

Mayer (*ibid.*, xiv, 1897, p. 29) makes a stronger stain by taking 2 grms. carmine, 5 grms. alum, and 100 c.c. water, and boiling for an hour, which sets some carminic acid free. The same result may be obtained by adding carminic acid to alum-carmine or carmalum.

Alum-carmine is an excellent stain. It is particularly to be recommended to the beginner, as it is easy to work with; it is hardly possible to overstain with it (except muscle). Its chief defect is that it is not very penetrating, and therefore quite unsuitable for staining objects of considerable size in bulk. This defect may, however, be to some extent overcome by employing the acid formula of Henneguy (§ 233), if it be not convenient to use Mayer's carmalum.

The stain is permanent in balsam; as to aqueous media I cannot say.

232. Cochineal Alum-carmine (Partsch, *Arch. mik. Anat.*, xiv, 1877, p. 180).—Powdered cochineal is boiled for some time in a 5 per cent. solution of alum, the decoction filtered, and a little salicylic acid added to preserve it from mould.

Another method of preparation has been given by CZOKOR (*ibid.*, xviii, 1880, p. 413). Mayer has carefully examined both, and finds that Partsch's is the more rational, the proportion of alum in it being exactly right, whilst in Czokor's it is insufficient. Partsch's fluid has also the advantage of keeping better.

RABL (*Zeit. wiss. Mik.*, xi, 2, 1894, p. 168) takes 25 grms. each of cochineal and alum, 800 c.c. of water, and boils down to 600 c.c.

These solutions give a stain that is practically identical with that of alum-carmine made from carmine, with perhaps even more delicate differentiations (but that depends much on the quality of the carmine, the quality of the cochineal, and the nature of the objects to be stained). They should be used in exactly the same way as the carmine fluid.

233. Acetic Acid Alum-Carmine (HENNEGUY, in *Traité des Méth. techn.*, LEE et HENNEGUY, 1887, p. 88).—Excess of carmine is boiled in saturated solution of potash alum. After cooling add 10 per cent. of glacial acetic acid, and leave to settle for some days, then filter.

For staining, enough of the solution is added to distilled water to give it a deep rose tint. In order to ensure rapid diffusion it is well to bring the tissues into the stain direct from alcohol. Stain for twenty-four to forty-eight hours, and wash for an hour or two in *distilled* water. Mount in balsam. You can mount in glycerin, but the preparations do not keep so well as in balsam.

The advantage of this carmine is that it has greater power of penetration than the non-acidified alum-carmine.

234. Alum-Carmine and Picric Acid.—Alum-carmine objects may be double-stained with picric acid. LEGAL (*Morph. Jahrb.*, viii, p. 353) combines the two stains by mixing ten vols. of alum carmine with one of saturated picric acid solution. I consider this to be a very recommendable practice.

235. Aceto-Carmine (Acetic Acid Carmine) SCHNEIDER (*Zool. Anzeig.*, 1880, p. 254).—To boiling acetic acid of 45 per cent. strength add carmine until no more will dissolve and filter. (Forty-five per cent.

acetic acid is, according to Schneider, the strength that dissolves the largest proportion of carmine.)

To use the solution you may either dilute it to 1 per cent. strength, and use the dilute solution for slow staining; or a drop of the concentrated solution may be added to a fresh preparation under the cover-glass. If you use the concentrated solution it *fixes* and stains at the same time, and hence may render service for the study of fresh objects. It is very penetrating, a quality that enables it to be used where ordinary reagents would totally fail. The stain is a pure nuclear one. Unfortunately the preparations cannot be preserved, and for this and other reasons the stain is *of very restricted applicability.*

A similar stain has been prepared with formic acid by PIANESE (see *Zeit. wiss. Mik.*, x, 4, 1894, p. 502). Probably for almost all the purposes for which aceto-carmine is useful, methyl-green will give better results.

For BURCHARDT's pyroligneous-acid carmines see *Arch. mik. Anat.*, liii, 1898, p. 232; and *Jena. Zeit. Naturw.*, xxxiv, 1900, p. 720.

236. Iron Carmine.—Valuable stains may be obtained by using iron as a mordant for carminic acid. I recommend trial of the following, which I have already published in the *Traité des Méth. Techniques,* LEE et HENNEGUY, 1902. Sections (I have not tried material in bulk) are mordanted (a few hours will suffice) in sulphate of iron (Benda's *Liquor ferri,* as for iron hematoxylin), washed, and stained for an hour or so in 0·5 per cent. solution of carminic acid in alcohol of 50 per cent. Wash in alcohol of 50 per cent.; no differentiation is necessary. When successful, an almost pure chromatin stain, quite as sharp as iron hæmatoxylin, but much less intense. I have not been able to get it strong enough for all purposes; there is a limit of intensity which it does not seem able to exceed. And with some objects I have failed to get a sharp stain.

237. Iron Carmine.—PFEIFFER VON WELLHEIM (*Zeit. wiss. Mik.,* xv, 1898, p. 123) mordants for six to twelve hours in a very weak solution of chloride of iron in 50 per cent. alcohol, washes in 50 per cent. alcohol, and stains for a few hours in a dilute solution of carminic acid in 50 per cent. alcohol. Overstains may be corrected with 0·1 to 0·5 per cent. HCl alcohol. I find this good, but not so good as the last.

238. Iron Carmine (ZACHARIAS, *Zool. Anz.*, 1894, p. 62).—Stain for several hours in an aceto-carmine (made by boiling 1 grm. of carmine with 150 to 200 c.c. of acetic acid of 30 per cent., for 20 minutes, and filtering). Rinse the objects with dilute acetic acid, and bring them (taking care not to touch them with metallic instruments if the aceto-carmine have been taken) into a 1 per cent. solution of ammoniated citrate of iron. Leave them, for as much as two or three hours if need be, till thoroughly penetrated and blackened (with sections this happens in a few minutes). Wash for several hours in distilled water.

This is at the same time a chromatin stain and a plasma stain. I consider it may render service in some cases.

239. Iron Carmalum (DE GROOT, *Zeit. wiss. Mik.*, xx, 1903, p. 21).—Dissolve 0·1 grm. of ferric alum in 20 c.c. distilled water and add 1 grm. carminic acid. Dissolve, add 180 c.c. of water, warm, add 5 grms. potash alum, dissolve, cool, filter, and add 2 drops of hydrochloric acid. To be used as carmalum, and said to give a stronger stain.

240. Iron Cochineal (SPULER, *Encyclopædie d. mik. Technik*, 1903, p. 153; other sources seem incorrect).—40 g. of cochineal is boiled with (how much—a litre ?) distilled water, and filtered after cooling. The residue is again boiled with water, filtered, and the filtrate added to the first filtrate, and evaporated down to 200 c.c. Add alcohol of 96 per cent. until a precipitate appears, decant, filter, and evaporate down to 400 c.c. For staining in bulk dilute two-fold (what with ?).

Stain for 48 hours in a stove, wash with water, put into solution of ferric alum of $\frac{3}{4}$ per cent. strength for 24 hours or more. If the stain is not sufficiently intense, the whole process may be repeated.

PETER (*Zeit. wiss. Mik.*, xxi, 1904, p. 314) takes 10 g. cochineal to 250 of water and evaporates down to 50 c.cm., then makes up to 150, filters, and adds 3 drops of hydrochloric acid for each 40 c.cm. of the filtrate. Decant after two days. Stain the material in bulk for 48 hours (sections 18 to 24) in an incubator, rinse, treat with iron-alum (§ 260) of $2\frac{1}{2}$ per cent. for one hour to one day (sections half to two minutes), wash, alcohol, xylol, paraffin, or balsam. Chromatin black, protoplasm grey, *yolk granules red*.

β. *So-called "Neutral" and Alkaline.*

241. Ammonia-Carmine.—In my opinion there is no valid excuse for using ammonia-carmine at all at the present day.

If, however, such a stain be used, care should be taken to get rid of the free ammonia as completely as possible. This is best done by the method of RANVIER. Make a simple solution of carmine in water with a *slight* excess of ammonia, and expose it to the air in a deep crystallising dish until it is entirely dried up. It should be allowed to putrefy if possible. Dissolve the dry deposit in pure water, and filter.

VAN WIJHE (*Vers. Akad.*, Amsterdam, viii, Deel, p. 507) takes an *old* strong solution of carmine in ammonia (or boils carmine with ammonia and peroxide of hydrogen), then precipitates it by adding alcohol to excess, washes the precipitate with alcohol, and dries it.

242. Magnesia-Carmine (MAYER, *Zeit. wiss. Mik.*, xiv, 1897, p. 23).—Take 1 grm. carmine, 0·1 grm. magnesia usta, and 50 c.c. distilled water, boil for five minutes, filter, and add three drops of formol. This is the *stock* solution. A *weak* solution may be made by boiling 0·1 grm. carmine for half an hour in 50 c.c. of magnesia water (made by leaving 0·1 grm. of magnesia usta in contact with 100 c.c. of spring water for a week with frequent agitation, and decanting when required for use). Said to be less injurious to tissues than the other alkaline carmines.

243. As to Picro-carmine.—The term "picro-carmine" is commonly used to denote a whole tribe of solutions in which carmine, ammonia, and picric acid exist *uncombined* in haphazard proportions. These solutions do *not* contain a double salt of picric and carminic acid and ammonia, or *picro-carminate of ammonia*. They are always alkaline, and frequently injurious to tissues. The *raison d'être* of picro-carmine does not lie in its capacity of affording a double stain. The double stain, if that is all that is wanted, can be just as well or better obtained by staining first with borax-carmine, or the like, and after-staining with picric acid. Its *raison d'être* is, that the picric acid in it is supposed to neutralise the ammonia.

Compare the paper of MAYER in *Zeit. wiss. Mik.*, xiv, 1897, p. 18.

244. MAYER'S **Picro-magnesia Carmine** (*ibid.*, 25) is relatively constant and innocuous to tissues. It consists of 1 vol. of the *stock* solution of magnesia-carmine (§ 242), and 10 vols. of a 0·6 per cent. solution of picrate of magnesia, or of equal parts of the *weak* solution and the picrate solution. The picrate may be obtained from GRÜBLER & HOLLBORN, or the solution may be made by heating 0·25 grms. of carbonate of magnesia in 200 c.c. of 0·5 per cent. solution of picric acid, allowing to settle and filtering.

245. Ranvier's Picro-carmine, Original Formula (*Traité*, p. 100). To a saturated solution of picric acid add carmine (dissolved in ammonia) to saturation. Evaporate down to one fifth the original volume in a drying oven, and separate by filtration the precipitate, pour in carmine, that forms in the liquid when cool. Evaporate the mother-liquid to dryness, and you will obtain the picro-carmine in the form of a crystalline powder of the colour of red ochre. It ought to dissolve completely in distilled water; a 1 per cent. solution is best for use.

For slow staining, dilute solutions may advantageously have 1 or 2 per cent. of chloral hydrate added to them.

Overstains may be washed out with hydrochloric acid, say 0·5 per cent., in water, alcohol, or glycerin.

Preparations should be mounted in balsam, or if in glycerin, this should be acidulated with 1 per cent. of acetic acid, or better, formic acid.

Ranvier's Newer Formula does not give a more constant product (see *previous editions*).

246. Van Wijhe dissolves 0·5 per cent. of the dry ammonia-carmine, § 241, in a 1 per cent. solution of neutral picrate of ammonia, boils until the vapour ceases to blue reddened litmus paper, and adds 1 per cent. of chloral hydrate. Gives an almost neutral preparation.

247. Other Formulæ for Picro-carmine.—I have tried most of them, and found no real advantage in any of them (see *previous editions*).

248. Other Aqueous Carmines (Acid and Alkaline).—For all of them see *previous editions.*

B. ALCOHOLIC CARMINE STAINS.

249. Alcoholic Borax-carmine (Grenacher, *Arch. mik. Anat.*, xvi, 1879, p. 466, *et seq.*).—Make a *concentrated* solution of carmine in borax solution (2 to 3 per cent. carmine to 4 per cent. borax) by boiling for half an hour or more; dilute it with about an equal volume of 70 per cent. alcohol, allow it to stand some time and filter. Or the mixture of carmine and borax solution is *allowed to stand for two or three days* and occasionally stirred; the greater part of the carmine will dissolve. To the solution is added an equal bulk of 70 per cent. alcohol; the mixture is allowed to stand for a week, and then is filtered. If on keeping more carmine is deposited, it must be refiltered.

Preparations should remain in the stain until they are thoroughly penetrated (for days if necessary), and then be brought (*without first washing out*) into alcohol of 70 per cent.

acidulated with 4 to 6 drops of hydrochloric acid to each 100 c.c. of alcohol. They are left in this until the stain is differentiated, and may then be washed or hardened in neutral alcohol. Four drops of HCl is generally enough. Three drops I find not quite sufficient. The stained objects should remain in the acidulated alcohol till they acquire a bright transparent look. This may require days.

For delicate objects, and for very impermeable objects, it may be well to increase the proportion of alcohol in the stain; it may conveniently be raised to about 50 per cent. It should not exceed 60 per cent. in any case (MAYER).

This stain used to be the most popular of any for staining in bulk. It is easy to use, and gives a most splendid coloration. But it is not so penetrating as is commonly supposed, and has the defect of sometimes forming precipitates in the cavities of bulky objects which cannot be removed by washing out. And the fluid is alkaline, and therefore may not be suitable for certain delicate work.

250. MAYER'S **Paracarmine** (*Mitth. Zool. Stat. Neapel*, x, 3, 1892, p. 491).—Carminic acid, 1 grm.; chloride of aluminium, 0·5 grm.; chloride of calcium, 4 grms.; 70 per cent. alcohol, 100 c.c. Dissolve cold or warm, allow to settle, and filter. A light red liquid, especially adapted for staining in bulk, and much like Grenacher's alcoholic borax-carmine.

Objects to be stained *should not have an alkaline reaction*, nor contain any considerable amount of carbonate of lime (spicules or skeletal parts of corals, etc.) which would give rise to precipitates. Wash out sections or objects intended to be sectioned, with pure 70 per cent. alcohol. Objects intended to be mounted whole may be washed out with a weak solution of aluminium chloride in alcohol, or if this be not sufficient, with 5 per cent. common acetic acid (or 2·5 per cent. glacial acetic acid) in alcohol. This may also be done with section material, if it is desired to obtain a more purely nuclear stain.

For staining bulky objects with large cavities, such as *Salpa*, the solution should be diluted (with alcohol); and as this may cause precipitates to form during the staining, especially if the objects are not very clean, it is advisable to *slightly acidify the dilute solutions.*

Paracarmine is less hurtful to delicate tissues than borax carmine; it is more highly alcoholic, therefore more penetrating; it has less tendency to form granular precipitates in the interior of objects, and generally keeps perfectly without precipitating.

251. Alcoholic Hydrochloric-Acid Carmine.—Sometimes it is desirable to possess a powerful staining medium more highly alcoholised than the foregoing, and of acid reaction.

GRENACHER'S receipt (*Arch. f. Mik. Anat.*, xvi, 1879, p. 468) is troublesome. That of MAYER (*Mitth. Zool. Stat. Neapel*, iv, 1883, p. 521; *Intern. Monatsschr. f. Anat., etc.*, 1897, p. 43) is better: Carmine 4 grms.; water, 15 c.c.; hydrochloric acid, 30 drops. Boil till the carmine is dissolved, add 95 c.c. of 85 per cent. alcohol, and neutralise by adding ammonia until the carmine begins to precipitate.

If it be desired to dilute the solution, it should be done with alcohol, not water, and alcohol of 80 to 90 per cent. should be taken for washing out.

If it be desired to have a purely nuclear stain, the alcohol must be *very slightly* acidulated with HCl.

For a complicated receipt of LOEWENTHAL see *Zeit. wiss. Mik.*, xix, 1902, p. 56.

252. Alcoholic Cochineal, MAYER'S Old Formula (*Mitth. Zool. Stat. Neapel.*, ii, 1881, p. 14).—Cochineal in coarse powder is macerated for several days in alcohol of 70 per cent. For each gramme of the cochineal there is required 8 to 10 c.c. of the alcohol. Stir frequently. Filter, and the resulting clear, deep red solution is fit for staining.

The objects to be stained must previously be saturated with alcohol of 70 per cent., and alcohol of the same strength must be used for washing out or for diluting the staining solution. The washing out must be repeated with fresh alcohol until the latter takes up no more colour. Warm alcohol acts more rapidly than cold. Overstaining seldom happens; it may be corrected by means of 70 per cent. alcohol, containing $\frac{1}{10}$ per cent. hydrochloric or 1 per cent. acetic acid.

Small objects and thin sections may be stained in a few minutes; larger animals require hours or days.

A nuclear stain, slightly affecting protoplasm. The colour varies with the reaction of the tissues, and the presence or absence of *certain salts* in them. Crustacea with thick chitinous integuments are generally stained red, most other organisms blue. The stain is also often of different colours in different tissue elements of the same preparation. Glands or their secretion often stain grey-green.

Acids lighten the stain and make it yellowish-red. Caustic alkalies turn it to a deep purple.

All acids must be carefully washed out from the objects before staining, or a diffuse stain will result. The stain is permanent in oil of cloves and balsam.

The high penetrating power of this fluid allows it to be employed in the case of organisms, such as Arthropoda, whose chitinous investments are but very slightly permeable by aqueous solutions.

It has over the new fluid (next §) the advantage of being more highly alcoholic ; and it does not contain free acid, so that it *can be used with calcareous structures* which it is wished to preserve—which the new fluid cannot. For specimens of *Pluteus*, for instance, I find it excellent.

253. Mayer's Alcoholic Cochineal, New Formula (*Mitth. Zool. Stat. Neapel*, x, 1892, p. 498).—Cochineal, 5 grms. ; chloride of calcium, 5 grms. ; chloride of aluminium, 0·5 grm. ; nitric acid of 1·20 sp. gr., 8 drops ; 50 per cent. alcohol, 100 c.c. Powder the cochineal finely and rub up in a mortar with the salts, add the alcohol and acid, heat to boiling-point, leave to cool, leave for some days standing with frequent agitation, filter.

Use as the old tincture, the objects being prepared and washed out with 50 per cent. alcohol. The stain is like that of paracarmine, but not quite so strong and not so sharp. Mayer only recommends it as a *succedaneum* of paracarmine.

Since this fluid contains in itself all the necessary salts (see § 227), it has over the old one the advantage of giving good results with *all classes of objects*, with the disadvantage of being less highly alcoholic.

CHAPTER XIII.

HÆMATEIN (HÆMATOXYLIN) STAINS.

254. *Introduction.—Hæmatoxylin* is a dye extracted from logwood. It is a substance that oxidises very readily, thus becoming converted into *hæmatein,* or, as often happens, into other more highly oxidised products. It appears to be now thoroughly well established (see Nietzki, *Chemie der organischen Färbstoffe,* Berlin, Springer, 1889, pp. 215—217, and Mayer, *Mitth. Zool. Stat. Neapel,* x, 1891, p. 170) that the colouring agent in solutions of logwood or hæmatoxylin is not the hæmatoxylin itself, but hæmatein formed in them (or, in some cases, one of the higher oxidation products).

Hæmatein is an acid body, a "colour acid" (§§ 216, 218). Substantively employed, it is a very weak plasma stain. But combined with appropriate mordants it becomes basophilous, and can be made to give a powerful nuclear stain, or at the same time a nuclear and a selective plasma stain. The mordants employed in histology are aluminium, chrome, iron, copper, vanadium, and molybdenum. Compounds of hæmatein with the other heavy or alkaline metals have been tried, but do not afford useful histological stains. Aluminium and iron are the mordants most employed, the former furnishing lakes used for progressive staining of material in bulk, the latter forming in most cases in the tissues a lake that requires differentiation, and is only applicable to the staining of sections.

The presence of a sufficient amount of hæmatein in staining solutions was formerly brought about by allowing solutions of hæmatoxylin to oxidate spontaneously by exposure to air. The change thus brought about in the solutions is known as "ripening," and until it has taken place the solutions are not fit to use for staining.

It was discovered by Mayer and Unna independently (see Mayer in *Mitth. Zool. Stat. Neapel,* x, 1891, pp. 170—186;

Unna in *Zeit. wiss. Mik.*, viii, 1892, p. 483) that nothing is easier than to bring about this change artificially; all that is necessary being, for instance, to add to a solution of hæmatoxylin containing alum a little neutralised solution of peroxide of hydrogen or other powerful oxidising agent. The solution becomes almost instantaneously dark blue, "ripe," and fit for staining. Other methods of "ripening," or of preparing hæmatein separately, are given further on, and constitute a great progress. For under the old practice of leaving staining solutions to "ripen" by the action of the air, it is necessary to wait for a long time before the reaction is obtained. During all this time, it may be weeks or months, there is no means, except repeated trial, of ascertaining whether the solution at any moment contains sufficient hæmatin to afford a good stain. And here a second difficulty arises: the oxidising process continuing, the solutions become "over-ripe"; the hæmatein, through further oxidation, passes over into colourless compounds, and the solutions begin to precipitate. They are therefore, in reality, a mixture in constantly varying proportions of "unripe," "ripe," and "over-ripe" constituents (the first and last being useless for staining purposes), and, in consequence, their staining power is very inconstant.

Logically, therefore, as concluded by Mayer, not hæmatoxylin, but *hæmatein*, should be taken in the first instance for making the staining solution. This at once relieves us from the tedious and uncertain process of "ripening" in the old way. We have a ripe solution to begin with, and we know that it must be ripe. (A discovery of Unna's, to be mentioned further on, affords a means of preventing the "over-ripening" brought about by excessive oxidation.)

If, however, it be still preferred to use hæmatoxylin (as may be indicated in some cases, *e. g.* the iron-hæmatoxylin process), this should not be done by dissolving the hæmatoxylin crystals straight away in the other ingredients of the staining solution. The solutions should be made up from a strong stock solution made by dissolving hæmatoxylin crystals in absolute alcohol: one in ten is a good proportion. This solution should be kept for a long time—months, at least, a year if possible; it gradually becomes of a vinous red, and should not be used till it has become quite dark. It has then become to a great extent oxidised into hæmatein, and the staining solutions made up from it will be at once fairly ripe.

255. Hæmatoxylin is found in commerce in the form of crystals, either colourless or browned by oxidation, easily soluble in either water, glycerin, or alcohol.

256. Hæmatein is found in commerce as a brown powder, entirely, though with difficulty, soluble in distilled water and in alcohol, giving a yellowish-brown solution, which remains clear on addition of acetic acid. Alkalies dissolve it with a blue-violet tint. MAYER (*Zeit. wiss. Mik.*, xx, 1903, p. 409) prepares it as follows : 1 grm. of hæmatoxylin is dissolved by boiling in not more than 10 c.c. of distilled water, and to the solution is added a hot solution of 0·2 grm. of iodate of sodium in about 2 c.c. of water. Mix well and cool by placing the recipient in cold water. After a couple of hours bring the mixture on to a filter, wash thereon with cold water to remove the excess of iodide of sodium that has formed, then dry the residue.

There is also found in commerce an ammonia-compound of hæmatein—*Hæmateïn-Ammoniak,* also known as *Hæmateinum crystallisatum*; this may be obtained in a sufficiently pure state from GRÜBLER & HOLLBORN.

This is somewhat more easily soluble in both water and alcohol than hæmatein is, and does quite as well for staining purposes. The histologist can easily prepare it for himself as follows :

257. Hæmateate of Ammonia (MAYER, *Mitth. Zool. Stat. Neapel*, x, 1891, p. 172).—Dissolve 1 grm. of hæmatoxylin with the aid of heat in 20 c.c. of distilled water, filter if necessary, add 1 c.c. of caustic ammonia (of 0·875 sp. gr.), and bring the purple liquid into a capsule of such dimensions that its bottom be covered to a depth of not more than half a centimetre. Let the liquid evaporate at the ordinary temperature and be protected from dust. The dry product will consist of hæmateate of ammonia, about equal in weight to the hæmatoxylin taken in the first instance. The evaporation should not be hastened by heat, as this may give rise to the formation of substances that are insoluble in alcohol. The preparation should not be touched, until it is dry, with any other instruments than such as are made of glass, porcelain, or platinum. The product is not of perfectly constant quality. It ought to dissolve easily in water or alcohol, and the solution should not become turbid on addition of acetic acid; if it does, it is over-oxidised.

258. Iron Hæmatoxylin, Generalities.—This method is due to Benda (*Verh. Phys. Ges.*, 1885–1886, Nos. 12, 13, 14; *Arch. Anat. Phys.*, 1886, p. 562; *third ed.* of this work, p. 365). He then mordanted for a few minutes or hours in solution of ferric alum (§ 260), stained for a few minutes in aqueous solution of hæmatoxylin, and differentiated for a few minutes in chromic acid of 1 : 2000 strength.

Later (*Verh. Anat. Ges.*, 1893, p. 161) he abandoned the mordanting with ferric alum, because he thought it gave rise to precipitates of iron oxide in the tissues, and took for a mordant a solution of persulphate of iron (next §) and differentiated either in acetic acid or in the mordant itself (adjective stains are generally soluble in the mordant which has served to form the lake).

The method was independently worked out about the same time by M. Heidenhain, who mordants in ferric alum and differentiates in the same (§ 260). The method is almost universally practised in the form given by Heidenhain, not on account of any essential difference between the two, for there is none (unless it be that Benda seems only to have used very short staining baths, whilst Heidenhain also used very prolonged ones, which does make a difference), but chiefly because Heidenhain has given more precise instructions concerning the process.

After carefully comparing Heidenhain's process with Benda's later process, I find that the two give an absolutely identical stain; that is to say, that if you mordant in Benda's *liquor ferri* and differentiate in the same you will get exactly the same effect as by mordanting in ferric alum and differentiating in the same. But you may vary the results somewhat by varying the differentiation. Benda has pointed out (*Verh. Anat. Ges.*, xv, 1901, p. 156) that you may differentiate either by an agent which simply dissolves the lake—such as acetic or hydrochloric acid; or by an oxidising agent, such as the above-mentioned chromic acid, or the *liquor ferri* or the ferric alum. The former, he thinks, are the best for the demonstration of nuclear structures, the latter for cytoplasmic structures. For these he greatly recommends WEIGERT'S borax-ferricyanide mixture, as being the easiest and safest to employ.

For myself, I find that differentiation in the iron salt (§ 259 or § 260) is sufficient for almost all purposes. Acetic acid of 30 per cent. acts much too quickly to be safe, and causes swelling of the tissues.

Van GIESON'S picro-säurefuchsin (§ 309) has been recommended as a differentiation fluid by Benda (*Deutsch. med. Wochenschr.*, 1898, No. 30). I find it gives very delicate differentiations, but acts very slowly, requiring nearly as many hours as the iron alum solution does minutes. The addition of the säurefuchsin to the picric acid is, I find, not necessary, and may prove an injurious complication. In these processes hæmatoxylin is generally used for the stain, *not hæmatein*, the iron salt oxidising it into hæmatein, or into a higher oxidation product. I have obtained some good stains with hæmatein, but also some very bad ones; presumably the solutions easily over-oxidise on contact with the iron salt.

The hæmatoxylin is generally dissolved in water. I frequently prefer alcohol, of 50 per cent., as less injurious to tissues.

The method is a regressive one. It has been proposed to stain progressively, which I have tried, and had extremely bad results.

The differentiation requires to be carefully timed. For this reason the method is not applicable to tissues in bulk, but only to sections, which should be thin, best not over 10 μ.

Iron hæmatoxylin is one of the most important of stains. It enables us to stain elements which cannot be selectively stained in any other way. The stain is very powerful, and of a certain optical quality that is peculiarly suited to the employment of high powers; it will allow of the use of deeper eye-pieces than other stains. It will take effect on any material, and is quite permanent. Further details as to the characters of the stain are given in § 260.

259. BENDA's **later Iron Hæmatoxylin** (*Verh. d. Anat. Ges.*, vii, 1, 1893, p. 161). Sections are mordanted for twenty-four hours in *liquor ferri sulphurici oxidati*, P.G.,* diluted with one or two volumes of water. They are then well washed, first with distilled water, then with tap water, and are brought into a 1 per cent. solution of hæmatoxylin in water in which they remain till they have become thoroughly black. They are then washed and differentiated. The differentiation may be done either in 30 per cent. acetic acid, in which case the progress of the decoloration must be watched; or in a weaker acid, which will not require watching; or in the sulphate solution strongly diluted with water.

I find that if the iron solution be taken for the differentiation, it should be taken *extremely* diluted (of a *very pale* straw-colour), and the progress of the differentiation watched; as if it be only diluted about tenfold, for instance, the decoloration is extremely rapid. See also last §.

I also find that Benda's mordant is unnecessarily, sometimes harmfully, strong, and that the *Liquor ferri* may be diluted *tenfold* with advantage. The duration of the bath in the mordant is also for most purposes excessive as directed by Benda. I find that three to six hours in the solution diluted tenfold is generally sufficient, with favourable material.

260. Iron Hæmatoxylin (M. HEIDENHAIN, "Über Kern und Protoplasma," in *Festschr. für Kölliker*, 1892, p. 118).—Sections are treated from half an hour to at most two or three hours with a 1·5 to 4 per cent. solution of ferric alum (ammonio-ferric sulphate). By this is always meant in histology the double salt of *ammonium* and sesquioxide of iron $(NH_4)_2Fe_2(SO_4)_4$, in clear violet crystals; the double salt of the protoxide, or salt of MOHR in green crystals, will not serve. If the crystals have become yellow and opaque, they have gone bad, and should be rejected. They ought to be kept in a stoppered bottle, and the solution should be made in the cold (*Arch. mik. Anat.*, xliii, 1894, pp. 431, 435). The sections are then washed with water and stained for half an hour in an aqueous solution (of about 0·5 per cent.) of hæmatoxylin. They are then rinsed with water, and again treated with the iron solution, which slowly washes out the stain. The progress of the differentiation ought to be controlled under the microscope. The sections should to this end be removed from time to time from the alum solution, and put into tap-water whilst they are being examined. This is favourable to the stain. As soon as a satisfactory differentiation has been obtained, the preparations are washed for at least a quarter of an hour, in running water, but not more than an hour, and mounted. The results differ according to the duration of the treatment with the iron and the stain. If the baths have been of short duration, viz. not more than half an hour in the iron and as much in the stain, *blue* preparations will be obtained. These show a very intense and highly differentiated stain of nuclear structures, cytoplasmic structures being pale. If the baths in the iron and in the stain have been prolonged (twelve to eighteen hours), and the subsequent differentiation in the second iron bath also duly prolonged, *black* preparations will result. These show chromosomes stained, "central corpuscles," stained intensely black, cytoplasm sometimes colourless, sometimes grey, in which case achromatic spindle-fibres and cell-plates are stained, connective tissue fibres black, red blood corpuscles black, micro-organisms sharply stained, striated muscle very finely shown.

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* This preparation consists of sulphate of iron, 80 parts; water, 40; sulphuric acid, 15; and nitric acid, 18, and contains 10 per cent. of Fe. Doubtless the *ferri persulphatis liquor* B. P., will do instead; the point is, to have a per-salt, and not a proto-salt.

Later (*Zeit. wiss. Mik.*, xiii, 1896, p. 186) Heidenhain gives further instructions for the employment of this stain in the study of "central corpuscles." All alcohol should be removed from the tissues,* by means of distilled water before bringing them into the mordant. This should be a 2½ per cent. solution of ferric alum, *not weaker*. Leave the sections therein (fixed to slides by the water method, § 200) for six to twelve hours, or at least not less than three. Keep the slides upright in the mordant, not lying flat. Wash out *well* with water before staining. Stain in a "ripened" hæmatoxylin solution, *i. e.* one that has stood for four weeks [of course if you make it up with the ripened brown alcoholic solution recommended § 254, *sub. fin.*, this will be superfluous]. Stain from twenty-four to thirty-six hours. *Use the same staining solution over and over again* until it becomes spoilt; for the solution after having been used gives a more energetic stain, owing to its containing a trace of iron brought over by the sections. Differentiate in a 2½ per cent. solution of ferric alum. Rinse for ten minutes in running water, clear with xylol, *not* with any essential oil, and mount in xylol-balsam. See also § 657.

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* Why? I find my iron-alum solution, as well as the *liquor ferri sulph. oxid.*, last §, mix clear with alcohol without the least precipitate forming.

GURWITSCH (*Zeit. wiss. Mik.*, xviii, 1902, p. 291) floods sections on the slide with mordant, warms on a water-bath till bubbles are given off or the mordant becomes turbid, then stains with the hæmatoxylin in the same way. The whole process takes about ten minutes.

HELD (*Arch. Anat. Phys.*, *Anat. Abth.*, 1897, p. 277) adds to the staining bath a very little of the iron-alum solution until a scarcely perceptible precipitate is produced. A dangerous practice.

FRANCOTTE (*Arch. Zool. Expér.*, vi, 1898, p. 200) mordants with *tartrate* of iron, MALLORY (*Journ. Exper. Med.*, v, 1900, p. 15) with *chloride*.

See also § 258.

261. Iron Hæmatoxylin (BÜTSCHLI, *Unters. über. mikroskopische Schäume u. das Protoplasma*, etc., 1892, p. 80).—Sections treated with a weak brown aqueous solution of ferric acetate, washed with water, and stained in 0·5 per cent. aqueous solution of hæmatoxylin. A blue-black or brown-black stain of extraordinary intensity, used by Bütschli for sections, 1 μ in thickness, of Protozoa.

262. WEIGERT'S Iron Hæmatoxylin for Nuclear Figures (*Allg. Zeit. f. Psychiatrie*, l, 1894, p. 245).—Sections of alcohol material, cut without imbedding, are put for half an hour into *Tinct. Ferri Acet. Rademacheri*, rinsed, stained for a quarter of an hour in 1 per cent. solution of hæmatoxylin in water, rinsed, and differentiated rapidly in 70 per cent. alcohol containing 1 per cent. of hydrochloric acid.

263. Weigert's Iron Hæmatoxylin for General Use (*Zeit. wiss. Mik.*, xxi, 1904, p. 1).—In this the iron and hæmatoxylin are combined in one solution. Mix one part of a 1 per cent. solution of hæmatoxylin in alcohol of 96 per cent. with one of a solution containing 4 c.c. of *liq. ferri sesquichlor.*, 1 c.c. of officinal hydrochloric acid (sp. gr. 1·124) and 95 of water. The mixture may be kept for some days (until it begins to smell of ether), but is best used fresh. Stain sections for a few minutes; no differentiation is necessary.

264. Janssens' Iron Hæmatoxylin (**"Hématoxyline noire"**; *La Cellule*, xiv, 1897, p. 207).—A similar mixture to that of Delafield, § 274, ferric alum being taken instead of ammonium alum, the rest as in Delafield's. A progressive stain, nuclear: for yeast cells.

265. Aluminium Hæmatein (Alum Hæmatoxylin) Generalities.—The iron lakes are those most used for staining *sections*, the aluminium lakes those most used for staining *in bulk*. The mordant and dye are generally combined in a single staining bath, giving a progressive stain. The stain is in different tones of blue or red according to the composition of the staining solution. Neutral or alkaline solutions give a blue stain; acid solutions give a red one. In order to *get a blue stain* in preparations that have come out red through the acidity of the staining bath, it is a common practice to treat them with weak ammonia, in the belief that the blue colour is restored by neutralisation of the acid that is the cause of the redness. According to Mayer, the ammonia acts not by neutralising the acid, but by precipitating the alumina, which carries down the hæmatein with it (if no alumina were present the colour would be purple, not blue).* The *same result* can generally be obtained by merely washing out with common tap-water, which is usually sufficiently alkaline, and can be obtained with certainty by treatment with bicarbonate of soda or acetate of soda or potash. And this is the preferable course, as ammonia is certainly a dangerous thing to treat delicate tissues with. Of course this is a different question from that of *neutralising* with an alkali tissues that have been treated with an acid to correct over-staining. Here the neutralisation may be indicated in the interest of the *preservation* of the stain.

Squire (*Methods*, p. 22) finds that sections can be blued in a few seconds by treatment with a 1 : 1000 solution of bicarbonate of soda in distilled water. Mayer holds that acetate of potash is the most inoffensive reagent to take; a strength of 0·5 to 1 per cent. may be taken.

Several of these solutions have a great tendency to over-stain. Over-stains may be corrected by washing out with weak acids (*e.g.* 0·1 to 0·2 or even 0·5 per cent. of hydrochloric acid, or with oxalic or tartaric acid), but this is not favourable to the permanence of the stain. Carnoy (*La Cellule*, xii, 2, 1897, p. 215) recommends iodised water. If acids be used, it is well to neutralise afterwards with ammonia or bicarbonate of soda (0·1 per cent.).

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* Fischer, in his *Fixirung, Färbung u. Bau des Protoplasmus*, pp. 156, 157, does not admit this explanation. He proposes another one of a highly speculative nature.

Bicarbonate of soda may be used for neutralisation with 70 per cent. alcohol as the vehicle (von Wistinghausen, *Mitth. Zool. Stat. Neapel*, x, 1891, p. 41 ; *Zeit. wiss. mik.*, x, 4, 1893, p. 480).

Over-staining may be avoided by staining very carefully and slowly in extremely dilute solutions. It should be noted that the purest chromatin stains are obtained by staining for a short time (sublimate sections half an hour, say) in solutions of *medium* strength, such as hæmalum diluted ten to twenty-fold with water. The stain obtained either with very strong solutions, or with the slow stain of the dilute solutions, is at the same time a plasma-stain, which of course may or may not be desired. Mayer, in the *Grundzüge*, p. 151, says that very dilute solutions will give a pure nuclear stain if they have been diluted with *alum-solution*, or have been *acidified.* Chromosmium material will not yield a pure chromatin stain unless it is very *fresh* ; it is consequently next to impossible to obtain the reaction with paraffin sections of such material ; they constantly give a plasma-stain in addition to the chromatin stain, which is not the case with sublimate material.

The stain is fairly permanent in balsam, but is very liable to fade a little, and may fade a great deal. If acids have been used after staining, great care should be taken to wash them out thoroughly before mounting. In aqueous media the stain cannot be relied on to keep (this refers to the old solutions : Mayer finds that his hæmatein preparations have kept well for at least some months in glycerin, if not acid, and, with certain precautions, in balsam). Turpentine-balsam should not be used (Mayer, *in litt.*).

Formulæ §§ 266 to 277 give *aqueous* solutions ; and §§ 278, 279 *alcoholic* ones.

266. Mayer's **Hæmalum, Original Formula** (Mayer, *Mitth. Zool. Stat. Neapel*, x, 1891, p. 172).—One grm. of the colouring matter (either hæmatein or the ammonia salt, §§ 256,

257) dissolved with heat in 50 c.c. of 90 per cent. alcohol, and added to a solution of 50 gr. of alum in a litre of distilled water. Allow the mixture to cool and settle, and filter if necessary. Or more recently (*Grundzüge*, p. 169), instead of dissolving the hæmatein or salt in alcohol, Mayer rubs it up in a mortar with a very little glycerin.

It is not necessary to conform exactly to the proportions given, and a rough and ready hæmalum solution may be at any time extemporised by adding a few drops of alcoholic solution of hæmatein to an alum solution of any desired strength.

A dark liquid of about the tint, at first, of borax-carmine, becoming more blue-violet with time. It stains equally well, *either at first, for it is ripe from the beginning, or later.* Concentrated, it stains sometimes almost instantaneously, or in any case very rapidly. Diluted twenty-fold with distilled water it will still stain through the tentacles of a *Tubularia* in an hour. (Spring water or tap-water containing lime must not be used for diluting; perhaps weak solution of alum in distilled water is the best means of all.) After staining, sections may be washed out either with distilled or common water. The solution is *admirable for staining in bulk*. Large objects will, however, require twenty-four hours' staining, and should be washed out for the same time (this should be done with 1 per cent. alum solution if a sharp nuclear stain be desired). All alum must be carefully washed out of the tissues before mounting in balsam; and it is well to blue the stain with tap-water or otherwise, § 265. The solution unfortunately does not keep perfectly, but precipitates and becomes weak with age. When this has occurred, it is well to withdraw the quantity required for staining from the middle of the stock solution by means of a pipette, which should be wiped outside before allowing the liquid to run out of it. The stain is generally a nuclear one; in any case such may be obtained by washing out with alum-solution. Mayer's preparations have kept well in glycerin (care being taken not to have it acid), also in balsam. It is to be noted that if oil of bergamot be used for clearing, it must be thoroughly removed by means of oil of turpentine before mounting, and that oil of cloves is dangerous. It is best (Mayer, *in litt.*) to use only xylol, benzol, or chloroform, and to mount in xylol-balsam or chloroform-balsam or benzol-balsam.

Hæmalum may be mixed with alum-carmine, Säurefuchsin, or the like, to make a double staining mixture; but it seems preferable to use the solutions in succession.

267. Mayer's **Hæmalum, Newer Formula** (*Zeit. wiss. Mik.*, xx, 1903, p. 409).—Hæmatoxylin, 1 grm.; water, 1 litre. Dissolve the hæmatoxylin in a little of the water by boiling, and add it to the rest of the water. Add 0·2 grm. of iodate of sodium and 50 grm. of alum, dissolve at the normal temperature and filter. Or dissolve the hæmatoxylin and iodate in a five per cent. solution of alum. The hæmatoxylin oxidises into hæmatein during the process, and the solution is "ripe" and may be used at once (see § 256).

This solution does not keep very well, but may be made more stable by adding chloral hydrate and citric (or acetic) acid; of the first a quantity equal to that of the alum, and of the second a quantity equal to that of the hæmatoxylin.

268. Mayer's **Acid Hæmalum** (*op. cit.*, § 266, p. 174).—This is hæmalum with 2 per cent. glacial acetic acid (or 4 per cent. common acetic acid). To be used as the last, washing out with ordinary water in order to obtain a blue-violet tint of stain. It is a perhaps even more precise nuclear stain, and the solution keeps better.

269. Unna's **Half-ripe Constant Stock Solution** (*Zeit. wiss. Mik.*, viii, 1892, p. 483).

Hæmatoxylin	1
Alum	10
Alcohol	100
Water	200
Sublimed sulphur	2

If the sulphur be added to the hæmatoxylin solution only when the latter has become somewhat strongly blue, *i. e.* after two or three days' time, the stage of oxidation attained by the solution will be fixed by the sulphur. The solution in this state may be used for staining, and according to Unna will remain "constant" in staining power. Mayer (*Mitth. Zool. Stat. Neapel*, xii, 1896, p. 309) finds that the sulphur process does not preserve the solutions for long, whilst for some unexplained reason the simple addition of *glycerin* does; see below, "GLYCHÆMALUM."

270. Mayer's **Glychæmalum** (*Mitth. Zool. Stat. Neapel*, xii, 1896, p. 310).—Hæmatein (or hæmateate of ammonia) 0·4 grm. (to be rubbed up in a few drops of glycerin in a mortar till it dissolves) ; alum, 5 grms.; glycerin, 30; distilled water, 70. The stain is *not purely nuclear*, but may be made so by washing out with alum solution or a weak acid (§ § 265, 266). The solution *keeps admirably*.

271. HANSEN's Solution (*Zool. Anz.*, 1895, p. 158).—See *fourth edition*. Hansen oxidises a mixture of alum and hæmatoxylin by means of permanganate of potash. I find it does not keep. See also MAYER in *Mitth. Zool. Stat. Neapel*, xii, 1896, p. 309, or the *Grundzüge*, p. 171.

272. HARRIS's Solution (*Micr. Bull.*, xv, 1898, p. 47; *Journ. App. Mic.*, iii, p. 777).—Alum-hæmatoxylin solution ripened by addition of mercuric oxide. MAYER (*Grundzüge*, 1901, p. 171) finds the formula "gives too much hæmatein."

273. Böhmer's Hæmatoxylin (*Arch. mik. Anat.*, iv, 1868, p. 345: *Aerzt. Intelligenzbl., Baiern*, 1865, p. 382).—Make (A) a solution of hæmatox. cryst. 1 part, alcohol (absolute) 12 parts and (B) alum 1 part, water 240. For staining, add two or three drops of A to a watch-glassful of B.

The alcoholic solution of hæmatoxylin ought to be old and dark (§ 254). I consider this stain to be of merely historical interest.

274. Delafield's Hæmatoxylin (*Zeit. wiss. Mik.*, ii, 1885, p. 288; frequently attributed erroneously to GRENACHER or PRUDDEN).—To 400 c.c. of saturated solution of ammonia-alum* add 4 grms. of hæmatox. cryst. dissolved in 25 c.c. of strong alcohol. Leave it exposed to the light and air in an unstoppered bottle for three or four days. Filter, and add 100 c.c. of glycerin and 100 c.c. of methylic alcohol (CH_4O). Allow the solution to stand until the colour is sufficiently dark, then filter and keep in a tightly stoppered bottle.

This solution keeps well,—it may be said to keep for years. It is well to allow it to ripen for at least two months before using it.

For staining, enough of the solution should be added to pure water to make a very dilute stain; and even then care should be taken not to leave objects too long in the fluid. It is an extremely powerful stain.

BÜTSCHLI (*Unters. üb. mikroscopische Schäume u. das Protoplasma*, etc., 1892; *Zeit. wiss. Mik.*, ix, 1892, p. 197) recommends, under the name of "acid hæmatoxylin," solution of Delafield very strongly diluted, and with enough acetic acid added to it to give it a decidedly red tint. This gives a sharper and more differentiated nuclear stain than the usual solution.

275. Ehrlich's Acid Hæmatoxylin (*Zeit. wiss. Mik.*, 1886, p. 150).—

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* Ammonia-alum dissolves in about 11 parts of water.

Water	100 c.c.
Absolute alcohol	100 „
Glycerin	100 „
Glacial acetic acid	10 „
Hæmatoxylin	2 grms.
Alum in excess.	

Let the mixture ripen in the light (with occasional admission of air) until it acquires a dark red colour. It will then *keep*, with constant staining power, *for years*, if kept in a well-stoppered bottle. Sections are stained in a few minutes. It is stated that the solution is also very appropriate for staining in bulk, as over-staining does not occur.

Of all the old formulæ I have tried, this is the one that has given me the sharpest chromatin stain.

Mann (*ibid.*, xi, 1895, p. 487) makes up this stain with an equal quantity of hæmatein instead of hæmatoxylin.

Mayer (*Grundzüge*, first edition, p. 154) finds that this is too much and makes the mixture overstain; 0·4 grm. of hæmatein is quite enough.

276. Burchardt's **Pyroligneous Acid Hæmatoxylin** (*Arch. mik. Anat.*, liii, 1898, p. 232) would seem to be superfluous at least.

277. Apáthy's **Hæmatein Mixture I A** (*Mitth. Zool. Stat. Neapel*, xii, 1897, p. 712).—Make (A) a solution of 9 per cent. alum, 3 per cent. glacial acetic acid, and 0·1 per cent. salicylic acid in water, and (B) a 1 per cent. solution of hæmatoxylin in 70 per cent. alcohol, preserved for six to eight weeks in a bottle not quite full. Mix one part of A with one of B and one of glycerin. The solution will keep for years, and stains either sections or material in bulk. Apáthy uses it for staining nerve "primitive-fibrils"; it is, therefore, not a purely nuclear stain.

278. Kleinenberg's **Hæmatoxylin** (*Quart. Journ. Micr. Sci.*, lxxiv, 1879, p. 208).—Highly irrational and very inconstant in its composition and its effects; see early editions; also the criticism of Mayer (*Mitth. Zool. Stat. Neapel*, x, 1891, p. 174), and that of Squire in his *Methods and Formulæ*, p. 25, and the alternative formulæ of Squire (*loc. cit.*) and of von Wistinghausen (*Mitth. Zool. Stat. Neapel*, x, 1891, p. 41).

279. Mayer's **Hæmacalcium** (*Mitth. Zool. Stat. Neapel*, x, 1891, p. 182).—Hæmatein (or hæmateate of ammonia, §§ 256, 257), 1 grm.; chloride of aluminium, 1 grm.; chloride of calcium, 50 grms.; glacial acetic acid, 10 c.c. (or common acetic acid, 20 c.c.); 70 per cent. alcohol, 600 c.c. Rub up finely together the first two ingredients, add the acid and alcohol, dissolve either cold or with heat; lastly add the chloride of calcium.

A reddish-violet liquid. If the objects stain in too red a tone they may be treated with a solution (of about 2 per cent.) of chloride of aluminium in 70 per cent. alcohol, or with a 0·5 to 1 per cent. solution of acetate of soda or potash in absolute alcohol; but washing with neutral alcohol will generally suffice.

The solution is not perfectly stable, but in course of time (*op. cit.*, 1892, p. 499) turns blue and precipitates. To avoid this the mixture should be made up in two separate bottles, each containing half of the alcohol and of the acid, and one containing besides all the calcium chloride, the other all the hæmatein and all the aluminium chloride, equal quantities being taken from each when required for staining.

With certain objects this solution does not penetrate well. This may be remedied by acidifying the solution, or, which is better, by leaving the objects for some time before staining in acid alcohol. Anyway objects ought NOT to have an alkaline reaction. If these precautions be taken, it will not be necessary to use acid for washing out. For some objects also (*e. g.* Hydroida) the penetrating effect is enhanced by diluting the solution with one third volume of glycerin, or by increasing the proportion of aluminium chloride up to about eight times that of the hæmatein.

The solution is not recommended as giving as good results as hæmalum,—as a stain it is distinctly inferior; and Mayer is of opinion that no alcoholic hæmatein solution can be made to give [illegible] precise a stain as the aqueous solutions. He recommends [illegible] merely as a substitute for Kleinenberg's (in cases in which [illegible] *alcoholic* hæmatein stain seems indicated), as being convenient, easy to prepare, and constant in its effects, none of which qualities belong to Kleinenberg's formula.

280. Other Alumina-Hæmatein Solutions.—A large number of suppressed receipts will be found given in the *earlier editions.*

281. R. HEIDENHAIN'S **Chrome Hæmatoxylin** (*Arch. mik. Anat.*, xxiv, 1884, p. 468, and xxvii, 1886, p. 383).—Stain for twelve to twenty-four hours in a $\frac{1}{3}$ per cent. solution of hæmatoxylin in distilled water. Soak the objects for the same length of time in a 0·5 per cent. solution of neutral chromate of potash, which should be changed, if necessary, several times. Wash out the excess of chromate with water.

The above is a slightly modified form of the original process, in which staining was done in a stronger hæmatoxylin solution (0·5 to 1 per cent.), and bichromate was used for washing out instead of neutral chromate. The more recent process gives a sharper chromatin stain.

Objects that have been fixed in corrosive sublimate ought to be very carefully washed out with iodine, or the like (see § 69), as neutral hæmatoxylin forms a black precipitate with any excess of sublimate that may remain in the tissues (see TORNIER, in *Arch. mik. Anat.*, 1886, p. 181).

The stain is black to grey. It is *a plasma-stain as well as a chromatin stain.*

The process is *adapted to staining in bulk.* You can decolour the objects to any extent by prolonging the washing in the chromate.

The method may be varied by washing out after staining with alum solution (1 per cent.) instead of a chromate. In this case the stain will be blue.

282. APÁTHY'S **Modification of Heidenhain's Process** (*Zeit. wiss. Mik.*, v, 1888, p. 47).—This is an *alcoholic* method. Stain in a 1 per cent. solution of hæmatoxylin in 70 or 80 per cent. alcohol. Wash out (for "thin" sections, *i. e.* sections of 10 to 15 μ, half the time of staining —for "thicker" sections of 25 to 40 μ twice the time of staining) in 1 per cent. solution of bichromate of potash in 70 to 80 per cent. alcohol.

The bichromate solution is conveniently prepared by mixing one part of a 5 per cent. aqueous solution with about four parts of 80 to 90 per cent. alcohol. The mixture should be made immediately before using, and should be kept from the light during the process of decoloration, and be changed for fresh several times during the process. After the differentiation of the colour has been accomplished, the objects should be thoroughly washed (in the dark) in several changes of 70 per cent. alcohol.

For celloidin series of sections, Apáthy (*ibid.*, 1889, p. 170) stains in the hæmatoxylin solution as above for ten minutes; then removes the excess of hæmatoxylin fluid from the sections by means of blotting-paper, and brings the series for five to ten minutes into 70 per cent. alcohol containing only a few drops of a strong (5 per cent.) solution of bichromate. This must be done in the dark. If the hæmatoxylin be not removed with blotting-paper as described, the celloidin will take the stain. The sections should appear steel-blue to steel-grey.

283. SCHULTZE'S **Chrome Hæmatoxylin** (*Zeit. wiss. Mik.*, xxi, 1904, p. 5).—The tissues to be fixed for twelve or more hours in a bichromate or chromic acid solution, preferably an osmium-bichromate mixture or liquid of Flemming. Then to be washed out for twenty-four hours in 50 per cent. alcohol in the dark and stained for twenty-four hours or more in 0·5 per cent. hæmatoxylin in alcohol of 70 per cent., then washed out in alcohol of 80 per cent. Imbed through chloroform or cedar oil, not bergamot oil.

284. Vanadium Hæmatoxylin (HEIDENHAIN, *Encyclop. mik. Technik.*, 1903, p. 518).—Add 60 c.c. of a 6 per cent. solution of hæmatoxylin to a 0·25 per cent. solution of vanadate of ammonium (quantity not stated; should be 30 c.c., see Cohn in *Anat. Hefte*, xv, 1895, p. 302). The mixture to be used after three or four days; it will not keep over eight days. To be used with sections of sublimate material. A strong plasma stain for special purposes, especially mucus glands. Chromatin ("basichromatin") is hardly stained, but "oxychromatin" (plastin) strongly.

285. Benda's Copper Hæmatoxylin (*Arch. mik. Anat.*, xxx, 1887, p. 49). See fourth edition. According to my experience, not to be compared with iron hæmatoxylin, and superfluous.

286. Mallory's Phospho-molybdic Acid Hæmatoxylin (*Anat. Anz.*, 1891, p. 375).—One part 10 per cent. phospho-molybdic acid solution, 1 part hæmatoxylin, 100 parts water, and 6 to 10 parts chloral hydrate. Let the solution ripen for a week in sunlight, and filter. Chiefly for central nervous system. Sections should be stained for from ten minutes to one hour, and washed out in two or three changes of 40 to 50 per cent. alcohol. It is necessary that the solution be *saturated* with hæmatoxylin in order to obtain the best results; if a good stain be not obtained at once, more hæmatoxylin must be added.

See also Ribbert (*Centralb. allg. Path.*, vii, 1896, p. 427; *Zeit. wiss. Mik.*, xv, 1898, p. 93).

Sargent (*Anat. Anz.*, xv, 1898, p. 214) quotes this stain, preceded by mordanting for twenty-four hours in 5 per cent. sulphate of copper, as Kenyon's.

Kodis (*Arch. mik. Anat.*, lix, 1901, p. 211) takes hæmatoxylin, 1 part; molybdic anhydride, 1·5; water, 100; H_2O_2, 0·5, or a crystal of HgO. For sections of nervous tissue.

For Mallory's phosphotungstic hæmatoxylin, see § 846.

CHAPTER XIV.

NUCLEAR STAINS WITH COAL-TAR DYES.

287. Introduction.—The majority of coal-tar dyes occur in the form of *salts*. The reason for this is that the free colour-bases or colour-acids are less soluble in water than their salts (§ 216). The dyes themselves are distinguished as "basic," "acid," or "neutral," as explained § 216. The basic dyes are those generally used for nuclear stains, the acid and neutral ones those generally used for plasma stains.

The coal-tar dyes are generally employed substantively (§ 218), mordants only being *intentionally* resorted to in special cases.

Very few coal-tar dyes give a precise nuclear or chromatin stain by the *progressive* method (§ 222). Two of them—methyl green and Bismarck brown—are pre-eminently progressive chromatin stains. Many of the others—for instance, safranin, gentian, and especially dahlia—may be made to give a progressive nuclear stain with fresh tissues by combining them with acetic acid; but in ninety-nine cases out of a hundred are not so suitable for this kind of work as the two colours first-named, which practically form a class apart.

Again, very few coal-tar dyes give a pure plasmatic stain (one leaving nuclei unaffected). The majority give a diffuse stain, which in some few cases becomes, by the application of the *regressive* method (§ 222), a most precise and splendid chromatin stain.

But plasma staining is generally done by the progressive method.

The basic anilin dyes were at one time greatly in vogue for the staining of chromatin in researches on the structure of nuclei. They have been little used for that purpose since the working out of the iron hæmatoxylin process, which gives a more energetic stain. But they may still be useful as a means of controlling the iron hæmatoxylin process, which frequently stains all sorts of things besides chromatin, which does not occur with the best tar colour stains.

The acid and neutral anilin dyes afford some of our best plasma stains.

I recommend—for staining nuclei of fresh tissues, methyl green; for staining nuclei of fixed tissues by the regressive method, safranin, for a red stain; and gentian violet or Thionin for a blue one; as a plasma stain for sections, Säurefuchsin; for entire objects, picric acid.

A. *Progressive Stains.*

288. Methyl Green.—This is the most common in commerce of the "anilin" greens. It appears to go by the synonyms of *Methylanilin green, Grünpulver, Vert Lumière, Lichtgrün*; these two last are in reality the name of another colour. When first studied by Calberla, in 1874 (*Morphol Jahrb.*, iii, 1887, p. 625), it went by the name of *Vert en cristaux*. It is commonly met with in commerce under the name of more costly greens, especially under that of iodine green. It is important not to confuse it with the latter, nor with aldehyde green (*Vert d'Eusèbe*), nor with the phenylated rosanilins, *Paris green*, and *Vert d'Alcali*, or *Véridine*.

Methyl green is the chloromethylate of zinc and penta-methyl-rosanilin-violet. It is obtained by the action of methyl chloride on methyl violet. The commercial dye always contains unconverted methyl violet as a consequence of defective purification. It is sometimes adulterated with anilin blue (soluble blue). It is also sometimes adulterated with a green bye-product of the manufacture—the chloride of nona-methyl-para-leukanilin (see BENEDIKT and KNECHT'S *Chemistry of the Coal-tar Colours*).

MAYER (*Mitth. Zool. Stat. Neapel*, xii, 1896, p. 312) says that the presence of the blue impurity can be demonstrated by placing a drop of the solution of the dye on filter paper and holding the green spot over a bottle of ammonia. If the methyl green is pure the spot will disappear; if not pure it will turn violet. He also says that the violet can be easily removed by agitating the aqueous solution with chloroform. FISCHER (*Fixirung, Färbung, u. Bau d. Protoplasmus*, p. 89) shakes up the solution in a burette with a little amyl alcohol, which quickly becomes violet and collects at the top, whence it may be decanted off and the operation repeated.

Methyl green is extremely sensitive to the action of alkalies. It is therefore important to use it only in *acidified* solutions, and to use only acid, or at least perfectly neutral fluids for washing and mounting.

This is an *extremely important histological reagent.* Its chief use is as a *chromatin* stain for *fresh, unfixed tissues.* For this purpose it should be used in the form of a strong aqueous solution containing a little acetic acid (about 1 per cent. in general). *The solutions must always be acid.* (If the tissues have been previously fixed with acetic acid you will not get a chromatin stain). The same applies to fixation with acetic acid sublimate : whilst pure sublimate will allow of a chromatin stain (BURCKHARDT, *La Cellule,* xii, 1897, p. 364). You may wash out with water (best acidulated) and mount in some acid aqueous medium containing a little of the methyl green in solution. The mounting medium, if aqueous, *must be acidulated.*

Employed in this way, with *fresh, unfixed* tissues, methyl green is a pure chromatin stain, in the sense of being a precise colour reagent for chromatin. For *in the nucleus* it stains nothing but chromosomes or chromatin elements ; it does not stain plasmatic nucleoli (unless indeed these contain chromatin, as may happen, for instance, with the nucleoli of ova), nor caryoplasm, nor achromatic filaments. *Outside* the nucleus it stains some kinds of cytoplasm and some kinds of formed material, especially glandular secretions (silk, for instance, and mucin). The chromatin elements are invariably stained of a bright green (with the exception of the nuclein of the head of some spermatozoa), whilst extra-nuclear structures are in general stained in tones of blue or violet. But this metachromatic reaction is probably due to the methyl-violet impurity, and is not obtained with a chemically pure methyl green.

Besides being a perfectly precise test for chromatin in the fresh nucleus, methyl green has other advantages. Staining is *instantaneous ;* overstaining never occurs. The solution is very penetrating, kills cells instantly without swelling or other change of form, and preserves their forms for at least some hours, so that it may be considered as a delicate fixative. It may be *combined without precipitating* with divers fixing or preserving agents. Osmic acid (0·1 to 1 per cent.) may be added to it, or it may be combined with solution of Ripart and Petit (this, by the way, is an excellent medium for washing out in and mounting in).

Alcoholic solutions may also be used for staining. They also *should be acidulated with acetic acid.*

The stain does not keep easily. It is difficult to mount it satisfactorily in balsam, because the colour does not resist alcohol sufficiently (unless this be charged with the colour). The resistance of the colour to alcohol is, however (at all events if it be used in the Ehrlich-Biondi combination), considerably increased by treating the sections for a few minutes with tincture of iodine before staining (M. Heidenhain).

Of preparations mounted with excess of colour in the usual aqueous media, I find the most fortunate only survive for a few months. Dr. Henneguy, however, writes to me that it keeps well in Brun's glucose medium.

Undoubtedly methyl green is one of the most valuable stains yet known. *It is the classical chromatin stain for fresh tissues.*

It was first pointed out, I believe, by Heschl (*Wiener med. Wochenschr.*, 2, 1879), that methyl green is a reagent for amyloid degeneration. His observations were confirmed by Curschmann (*Virchow's Arch.*, vol. lxxix, 1880, p. 556), who showed that it colours amyloid substance of an intense violet; but this, as pointed out by Squire, *Methods and Formulæ*, etc., Churchill, 1892, p. 37) is undoubtedly due to its containing methyl violet as an impurity.

289. Bismarck Brown (Manchester Brown, Phenylen Brown, Vesuvin, La Phenicienne).—A fairly pure nuclear stain that will work either with fresh tissues or with such as have been hardened in chromic acid, or otherwise.

The colour is not very easily soluble in water. You may boil it in water, and filter after a day or two (Weigert, in *Arch. mik. Anat.*, xv, 1878, p. 258). You may add a little acetic or osmic acid to the solution. Maysel (*ibid.*, xviii, 1880, pp. 237, 250) dissolves the colour in acetic acid (this solution does not give a permanent stain). Alcoholic solutions may also be used, *e. g.* saturated aqueous solution diluted with one third volume of 90 per cent. alcohol; or Calberla's glycerin-and-alcohol mixture, or dilute glycerin (say of 40 per cent. to 50 per cent.) may very advantageously be employed.

The watery solutions must be frequently filtered. The addition to them of carbolic acid has been recommended (vide *Journ. Roy. Mic. Soc.*, 1886, p. 908). Bismarck brown stains rapidly, but never overstains. The stain is permanent both in balsam and in glycerin.

This colour may be used as a chromatin stain for fresh tissues in the same way as methyl green. Its stain is less precise, but stronger.

The chief use of this colour is for progressive staining; but it may be employed for staining by the regressive method (see § 301), and also for *intra-vitam* staining (§ 221) (for this purpose it is necessary to see that the colour employed be pure *and neutral*).

290. Methyl Violet (Methylanilin Violet, Anilin Violet, Paris Violet).—GRASER (*Deutsche Zeit. Chirurgie*, xxvii, 1888, pp. 538—584; *Zeit. wiss. Mik.*, v, 3, 1888, p. 378) recommends the following process:

Sections are stained for from twelve to twenty-four hours in a (presumably aqueous) solution so dilute that at the end of that time the sections will have taken up all the colour from the liquid. They are then washed out for a short time in acidulated alcohol, and then in pure alcohol (followed presumably by clearing and mounting in balsam). SCHIEFFERDECKER, whose account is here quoted, says that the results, as regards nuclear figures, are even finer than with safranin. The method is applicable to objects fixed in Flemming's mixture.

291. Other Progressive Stains.—Most of the basic tar colours used for regressive staining will also give by the progressive method a nuclear stain of greater or less purity if used in solutions acidified with acetic acid. Amongst these may be mentioned thionin, which need not even be acidified; also, for fresh tissues especially, gentian violet and dahlia.

B. *Regressive Stains.*

292. The Practice of Regressive Staining; the Staining Bath.—*Sections only*, or material that is thin enough to behave like sections, such as some membranes, can be stained by this method.

The solutions employed are made with alcohol, water, or anilin, or sometimes other menstrua, according to the solubility of the colour. There seems to be no special object in making them with alcohol if water will suffice, the great object being to get *as strong a solution as possible*. Indeed, the solutions made with strong alcohol are found not to give quite such good results as those made with water or weak alcohol. Alcohol of 50 per cent. strength, however, may be said to constitute a very generally desirable medium. The sections must be *very thoroughly* stained in the solution. As a general rule they cannot be left too long in the staining fluid. With the powerful solutions obtained with anilin a few minutes or half an hour will usually suffice, but to be on the safe side it is frequently well to leave the sections twelve to twenty-four hours in the fluid. Up to a certain point the more the tissues are stained the better do they resist the washing-out process, which is an advantage.

Material fixed in chromic or chromo-osmic mixtures gives a *sharper and more selective* stain than material fixed in sublimate or the like. In fact, *to ensure the best results, only material fixed in chromic mixtures* (or Hermann's fluid) *should be employed.*

During the staining the tissues become *overstained,* that is, charged with colour in an excessive and diffuse manner. The stain must now be *differentiated* by removal of the excess of colour.

293. Differentiation.—This is generally done with alcohol, sometimes *neutral,* sometimes *acidulated* (with HCl). The stained sections, if loose (celloidin sections), are brought into a watch-glassful of alcohol; if mounted in series on a slide they are brought into a tube of alcohol (differentiation *can* be done by simply pouring alcohol on to the slide, but it is better to use a tube or other bath). It is in either case well to *just rinse* the sections in water, or even to wash them well in it, before bringing them into alcohol.

The sections in the watch glass are seen to give up their colour to the alcohol in clouds, which are at first very rapidly formed, afterwards more slowly. The sections on the slide are seen, if the slide be gently lifted above the surface of the alcohol, to be giving off their colour in the shape of rivers running down the glass. In a short time the formation of the clouds or of the rivers is seen to be *on the point of ceasing*; the sections have become *pale* and somewhat *transparent,* and (in the case of chrom-osmium objects) have *changed colour,* owing to the coming into view of the general ground colour of the tissues, from which the stain has now been removed. (Thus chrom-osmium-safranin sections turn from an opaque red to a delicate purple.) At this point the differentiation is complete, or nearly so, and the extraction of the colour by the alcohol *must be stopped* (see § 294).

It is generally directed that absolute alcohol be taken for differentiation. This may be well in some cases, but in general 95 per cent. is found to answer perfectly well.

The hydrochloric-acid-alcohol extracts the colour *much more quickly from resting nuclei than from kinetic nuclei.* Therefore, washing out should be done with neutral alcohol whenever it is desired to have resting nuclei stained as well as dividing nuclei; the acid process serving chiefly to differentiate karyokinetic figures.

The proportion of HCl with which the alcohol should be acidified for the acid process should be about 1 : 1000, or less; seldom more.

The length of time necessary for differentiating to the precise degree required varies considerably with the nature of the tissues and the details of the process employed; all that can be said is that it generally lies between thirty seconds and two minutes. The acid process is *vastly more rapid* than the neutral process, and therefore of course more risky.

There exists also a method of differentiation known as *substitution*—one stain being made to wash out another. Thus methylen blue and gentian violet are discharged from tissues by aqueous solution of vesuvin or of eosin; fuchsin is discharged from tissues by aqueous solution of methylen blue. The second stain "substitutes" itself for the first in the general "ground" of the tissues, leaving, if the operation has been successfully carried out, the nuclei stained with the first stain, the second forming a "contrast" stain. In the paper of RESEGOTTI in *Zeit. wiss. Mik.*, v, 1888, p. 320, it is stated as a very general rule that colours that do *not* give a nuclear stain by the regressive method will wash out those that *do*. But RESEGOTTI's experiments do not seem to me to constitute a case in point. For he used the second colour, if I understand him rightly, in *alcoholic* solution; so that it remains uncertain how far the differentiation should be attributed to the second colour itself, and how far to the alcohol used as a vehicle. The same remark applies to BENDA's Safranin-and-Lichtgrün process.

294. Clearing.—After due differentiation, the extraction of the colour may be stopped by putting the sections into water; but the general practice is to clear and mount them at once.

You may clear with clove oil, *which will extract some more colour* from the tissues. Or you may clear with an agent that does not attack the stain (cedar oil, bergamot oil, xylol, toluol, etc.; see the chapter on Clearing Agents). If you have used neutral alcohol for washing out, you had perhaps better clear with clove oil, as neutral alcohol does not always, if the staining have been very prolonged, extract the colour perfectly from extra-nuclear parts. But if you have not stained very long, and if you have used acidulated alcohol for washing out, clove oil is not necessary, and it may be better not to use it, as it somewhat impairs the brilliancy of the stain. A special property of clove oil is that it helps to differentiate karyokinetic figures, as it *decolours resting nuclei more rapidly than those in division.*

Some colours are much more sensible to the action of clove oil than others; and much depends on the quality of this much-adulterated essence. New clove oil extracts the colour more quickly than old.

Series of sections on slides are conveniently cleared by pouring the clearing agent over them.

After clearing you may either mount at once in damar or balsam; or, stop the extraction of the colour, if clove oil have been used, by putting the sections into some medium that does not affect the stain (xylol, cedar oil, etc.). Chloroform should be avoided, either as a clearer or as the menstruum for the mounting medium.

295. General Results.—The results depend in great measure on the previous treatment of the tissues. If you have given them a prolonged fixation in Flemming's *strong* chromoaceto-osmic mixture, and have differentiated after staining with acid alcohol and cleared with clove oil, you will get, with some special exceptions, nothing stained but nucleoli and the chromatin of *dividing* nuclei, that of resting nuclei remaining unstained. If you have given a lighter fixation, with Flemming's weak mixture or some other fixing agent not specially inimical to staining, and have differentiated after staining with neutral alcohol, you will get the chromatin of *resting* nuclei stained as well. Either process may also stain mucin, the ground-substance of connective tissues (especially cartilage), the bodies of Nissl in nerve-cells, and the yolk of ova.

296. HENNEGUY'S **Permanganate Method** (*Journ. de l'Anat. et de la Physiol.*, xxvii, 1891, p. 397).—Permanganate of potassium is a mordant for many anilin dyes, and will enable a good stain to be procured in cases in which the usual methods fail.

Sections are treated for five minutes with 1 per cent. solution of permanganate of potassium. They are then washed with water and stained (for about half the time that would have been taken if they had not been mordanted with the permanganate) in safranin, rubin, gentian violet, vesuvin, or the like, and are differentiated with alcohol, followed by clove oil in the usual way. The stain is either purely nuclear, or in part plasmatic, according to the extent of the differentiation.

The mordanting action of permanganate of potassium on anilin dyes is so energetic that if it has been overmuch prolonged before staining with safranin, or, still more, with rubin, it becomes almost impossible to differentiate the sections properly; it may be necessary to leave them for a month or more in clove oil.

297. OHLMACHER'S **Formaldehyde Process** (*Medical News*, February 16th, 1895).—Ohlmacher states that formaldehyde is a powerful mordant for tar colours. Tissues may either be mordanted separately by treatment for a short time (one minute is enough for cover-glass preparations) with a 2 per cent. to 4 per cent. formalin solution; or the formalin may be combined with the stain. One gramme of fuchsin or methylen blue dissolved in 10 c.c. of absolute alcohol may be added to 100 c.c. of 4 per cent. formalin solution. Sections are said to stain in half a minute and to resist alcohol much more than is the case with those treated by the usual solutions.

298. Safranin.—One of the most important of these stains, on account of its great power, brilliancy, and superior permanence in balsam, and also on account of the divers degrees of electivity that it displays for the nuclei and other constituent elements of different tissues.

The great secret of staining with safranin is *to get a good safranin.* It is needful here to insist most urgently on what was said above (§ 224). Before thinking of working with this important reagent you should go to Grübler & Hollborn or to Münder, and order the safranin you want, specifying whether you want it for staining nuclei or for staining elastic fibres, or for what other purpose you may require it.

There are presumably at least a score of sorts of safranin in the market, differing to a considerable extent in colour, weight, solubility, and histological action. Some are easily soluble in water and not so in alcohol, some the reverse, and some freely soluble in both. Fourteen brands, supplied by Grübler and by Münder, have been studied by RESEGOTTI (*Zeit. wiss. Mik.*, v, 3, 1888, p. 320). Resegotti obtained his best results with the brands "Safranin wasserlöslich," "Safranin spirituslöslich," "XX," "XXBN," "TB," furnished by Grübler, and with the brands "Rein," "O," "FII," and "Conc.," supplied by Münder.

The brand I have been using for a long time, and which gives good results, is the "Safranin O" of Grübler & Co. As the processes of manufacture are constantly changing, the properties of the products are sure to vary somewhat from time to time.

Staining.—The majority of safranins are not sufficiently soluble in water, so that solutions in other menstrua must be employed.

PFITZNER (*Morph. Jahrb.*, vi, p. 478, and vii, p. 291) advised a solution of safranin 1 part, absolute alcohol 100 parts, and water 200 parts, the last to be added only after a few days.

FLEMMING (*Arch. mik. Anat.*, xix, 1881, p. 317) used a concentrated solution in absolute alcohol, diluted with about one half of water.

BABES (*ibid.*, 1883, p. 356) used (A) a mixture of equal parts of concentrated alcoholic solution and concentrated aqueous solution (this is very much to be recommended), or (B) a concentrated or supersaturated aqueous solution made with the aid of heat.

Some people still employ simple aqueous solutions.

The anilin solution of BABES (*Zeit. wiss. Mik.*, iv, 1887, p. 470) consists of water 100 parts, anilin oil 2 parts, and an excess of safranin. The mixture should be warmed to from 60° to 80° C., and filtered through a wet filter. This solution will keep for a month or two.

ZWAARDEMAKER (*ibid.*, iv, 1887, p. 212) makes a mixture of about equal parts of alcoholic safranin solution and anilin water (saturated solution of anilin oil in water;—to make it, shake up "anilin oil," which is nothing but pure anilin, with water, and filter). This, I find, will keep for many months, perhaps indefinitely.

I myself use equal parts of saturated solution in anilin water, and saturated solution in absolute alcohol.

Differentiation.—For general directions see §§ 293 and 294.

Flemming's *acid* differentiation (*Zeit. wiss. Mik.*, i, 1884, p. 350).—Differentiate, until hardly any more colour comes away, in alcohol acidulated with about 0·5 per cent. of hydrochloric acid, followed by pure alcohol and clove oil. (You may use the HCl in watery solution if you prefer it.) Or you may use a lower strength, viz. 0·1 per cent. at most (see *Arch. mik. Anat.*, xxxvii, 1891, p. 249); and this I find is generally preferable.

Objects are supposed to have been well fixed—twelve hours at least—in the *strong* chromo-aceto-osmic mixture, and stained for some hours. In this way you get kinetic chromatin and nucleoli alone stained.

Podwyssozki (*Beitr. z. Path. Anat.*, i, 1886; *Zeit. wiss. Mik.*, iii, 1886, p. 405) differentiates (for from a few seconds to two minutes) in a strongly alcoholic solution of picric acid, followed by pure alcohol. Same results (except that the stain will be brownish instead of pure red).

Babes recommends, for sections stained in the anilin solution, treatment with iodine, according to the method of Gram (see next section). This process has also been recommended by Prenant (*Int. Monatsschr. Anat.*, etc., iv, 1887, p. 368).

It has been shown by Ohlmacher (*Journ. Amer. Med. Assoc.*, vol. xx, No. 5, February 4th, 1893, p. 111) that if tissues be treated with solutions containing iodine or picric acid after staining with safranin, there may be produced in the tissue elements a precipitate of a dark red substance of a crystalline nature, but of lanceolate, semilunar, falciform, or navicellar forms. The precipitate is formed both in normal and pathological tissue, readily in carcinomatous tissues; and Ohlmacher concludes that many of the bodies that have been described as "coccidia," "sporozoa," or other "parasites" of carcinoma are nothing but particles of this precipitate.

See also the differentiation process of Martinotti and Resegotti (*Zeit. wiss. Mik.*, iv, 1887, p. 328) for alcohol-fixed material; and of Garbini (*Zeit. wiss. Mik.*, v, 2, 1888, p. 170).

In preparations made with chromo-aceto-osmic acid, safranin stains, besides nuclei, elastic fibres, the cell bodies of certain horny epithelia, and the contents of certain gland-cells (mucin, under certain imperfectly ascertained conditions).

The stain is perfectly permanent.

299. Gentian Violet may be used in aqueous solution, or as directed for safranin.

In some cases it may be useful to employ the method devised by GRAM for the differentiation of bacteria in tissues (*Fortschr. d. Medicin.*, ii, 1884, No. 6; *British Med. Journ.*, Sept. 6th, 1884, p. 486; *Journ. Roy. Mic. Soc.* [N.S.], iv, 1884, p. 817).

In Gram's method the sections are treated, after staining, with a solution composed of—

Iodine	1 gramme,
Iodide of potassium . .	2 grammes,
Water	300 „

for two or three minutes, until they become black. They are then differentiated with neutral alcohol, until they turn grey, and are then finally differentiated with clove oil.

By this process, in resting nuclei the nucleoli alone are stained, or the chromatin if stained is pale; in dividing nuclei the chromatin is stained with great intensity, being nearly black in the equatorial stage.

Gentian violet is an exceedingly powerful stain, quite as precise as safranin.

The stain keeps well. It is more or less dichroic, possibly owing to the fact that the dye is not a pure substance, but a mixture of "Krystallviolett" and methyl violet.

HERMANN (*Arch. mik. Anat.*, xxxiv, 1889, p. 58) first stains for twenty-four hours or more in safranin, differentiates incompletely with alcohol, then stains for three to five minutes in the anilin-water gentian solution, treats with the iodine solution for one to three hours, and finally differentiates with absolute alcohol.

300. Thionin.—The hydrochloride of thionin, or violet of Lauth, is a colour chemically nearly allied to methylen blue. I have classed it here as a regressive stain, but its action is so selective from the first that it may almost be considered to be a progressive stain. If you stain for only a short time (a few minutes) in a concentrated aqueous solution, hardly anything but the chromatin will be found to be stained. If the staining be prolonged, plasmatic elements will begin to take up the colour. After a short stain no special differentiation is required; all that is necessary is to rinse with water, dehydrate, and mount. After a strong stain you differentiate with alcohol in the usual way, with this advantage, that the stain is so highly resistent to alcohol that there is no risk whatever of overshooting the mark; the stain will not be more extracted in an hour than that of gentian or dahlia is in a minute, so that the process may be controlled under the microscope if desired. For this reason I think this stain may be useful to beginners, but I myself prefer gentian. It is a very powerful stain.

Thionin is a specific stain for mucin, *q. v.* Some observers have found the stain to fade. Wolff (*Zeit. wiss. Mik.*, xv, 1899, p. 312) says that, to avoid this, preparations should be mounted in a little solid colophonium or balsam melted over a flame.

301. Other Regressive Stains.—The foregoing, I think, may suffice for most practical purposes, but the following may be mentioned:

Dahlia, according to Flemming (*Arch. mik. Anat.*, xix, 1881, p. 317), best used in aqueous solution, either neutral or acidified with acetic acid, and differentiated with neutral alcohol. A pure blue stain, which keeps well.

Victoria Blue (Victoriablau) (Lustgarten, *Med. Jahrb. k. Ges. d. Aerzte zu Wien*, 1886, pp. 285—291).—This dye ("Victoriablau 4 A") has a special affinity for *elastic fibres.* For this object Lustgarten recommends an alcoholic solution of the dye diluted with two to four parts of water. Fixation in chrom-osmium, or at least in a chromic mixture, is, I believe, a necessary condition to this reaction. And you must stain for a long time.

Victoria has also a special affinity for *mucus-cells,* from which it is not washed out by alcohol.

With **Toluidin Blue** I have had some superb stains of chromatin, unfortunately accompanied by a diffuse staining of cytoplasm.

Mann (*Zeit. wiss. Mik.*, xi, 1894, p. 489) states that he has had good results by staining with it after eosin.

See further as to the micro-chemical properties of this dye, Harris, *The Philadelphia Medical Journal*, May 14th, 1898. It much resembles methylen blue.

Magdala Red (Naphthalin Red, Rose de Naphthaline).

Fuchsin (meaning the basic fuchsins, a series of Rosanilin salts having very similar reactions, and found in commerce under the names of Fuchsin, Anilin Red, Rubin, Rosein, Magenta, Solferino, Corallin).—Graser (*Deutsche Zeit. Chirurgie*, xxvii, 1888, pp. 538—584; *Zeit. wiss. Mik.*, v, 1888, p. 378) stains for twelve to twenty-four hours in a dilute aqueous solution, washes out for a short time in alcohol, stains for a few minutes in aqueous solution of methylen blue, and dehydrates with alcohol. A double stain. Chromatin and nucleoli red, all the rest blue.

Ziehl's **Carbolic Fuchsin** (from *Zeit. wiss Mik.*, vii, 1890, p. 39).

The stain is made either by taking—

Fuchsin	1 gramme,
Acid. carbol. crist. . .	5 grammes,
Alcohol	10 „
Aq. dest.	100 „

or by saturating a 5 per cent. aqueous solution of carbolic acid with concentrated alcoholic solution of fuchsin (the saturation of the carbolic solution with fuchsin is made manifest by the formation of a metallic-looking pellicle on the surface of the liquid). The stain is differentiated with alcohol followed by clove oil.

Kresofuchsin (Röthig, *Arch. mik. Anat.*, lvi, 1900, p. 354) is apparently the same substance as that obtained from fuchsin and resorcin by Weigert (see § 698). Its aqueous solution is red, and stains mucus, cartilage, keratin, and nuclei red, whilst its alcoholic solution is blue and stains elastin blue. See § 696.

Bismarck Brown has this advantage, that being sufficiently resistent to alcohol it may be utilised for staining entire objects.

Kaiser (*Biblioth. Zool.*, H. 7, 1 Hälfte, 1891; *Zeit. wiss. Mik.*, viii, 1891, p. 363) stains for forty-eight hours, and at a temperature of 60° C. in saturated solution of Bismarck brown in 60 per cent. alcohol (the solution to be made in boiling alcohol), and washes out (until all is decoloured except the karyokinetic figures) in 60 per cent. alcohol, containing 2 per cent. hydrochloric acid or 3 per cent. acetic acid.

Methyl Violet.

Benzoazurin (Martin, *Zeit. wiss. Mik.*, vi, 3, 1889, p. 193). Stain for an hour or so in dilute aqueous solution, and wash out with HCl alcohol.

Methylen Blue.

Nigrosin (Errera *Proc.-Verb. Soc. Belge de Mic.*, 1881, p. 134). The stain resists alcohol well.

CHAPTER XV.

PLASMA STAINS* WITH COAL-TAR DYES.

302. Introduction.—By a plasma stain is generally meant, rather vaguely, one that stains the extra-nuclear parts of cells and the formed material of tissues, or one of these. To be precise the class ought to be subdivided, and we ought to speak of cytoplasm stains, granule stains, ground-substance stains, or the like. But the vague general sense of the term will be sufficient for the purposes of the present chapter.

The plasma stains described in this chapter are for the most part—especially the most important of them—those obtained by means of "acid" dyes (§ 216); but some of them are obtained by means of "neutral" dyes (§ 216), and a few by "basic" dyes.

The mode of staining is generally progressive, almost always so when acid colours, used substantively (§ 218), are employed. But the regressive method, with differentiation, is sometimes made use of, especially when a mordant has been used with the dye.

In some processes, *e.g.* Flemming's orange method, a basic and an acid dye (or *vice versâ*) being employed in succession, there is formed *in the tissues* a neutral colour (§ 216) which effects the desired stain. These may be considered as adjective stains, the first colour serving as a mordant for the second. Not any two dyes taken at haphazard will behave in this way: they must be such as to form by combination a *suitable* neutral lake. The basic dye may be made the primary stain, as in Flemming's process: or the contrary, according to the properties of the dyes employed, as in §§ 317, 328, 335.

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* This chapter includes only such stains as are used in *ordinary* work on tissues in bulk or sections, stains for *special* purposes being treated under "Nervous tissue," "Blood," etc. It includes some double or triple stains that affect nuclei as well as plasma, but in different hues.

In such stains as Reinke's orange method, or the Ehrlich-Biondi mixture, and many others, one or more neutral colours are formed *in the mixture* and stain progressively. In general the method of mixtures is to be preferred, as being less capricious in its results than that of successive stains. I am not acquainted with any plasma stain that is thoroughly satisfactory for delicate work. I recommend for sections Säurefuchsin, either alone or in the form of Ehrlich-Biondi mixture, or Ehrlich's triacid: for material in bulk, picric acid (but only for rough work).

303. Säurefuchsin (Acid Fuchsin, Fuchsin S, Acid Rubin, Rubin S, Säurerubin, Acid Magenta, Magenta S).—The chemical description of this acid colour has been given (§ 216): it is important not to confound it with basic fuchsin, as seems to have been done by some writers.

This dye is highly soluble in water, less so in alcohol. I use a 0·5 per cent. solution in water and allow it to act on sections for a few minutes in the case of easily stainable material, or twenty-four hours or more for chrom-osmium material. The stain is fast to neutral alcohol. It is very sensitive to alkalies, so that overstains can easily be removed by washing for a few minutes in tap-water. Acids strengthen the stain, so that it is frequently useful to treat sections after staining for a few seconds with acidulated water. A good stain should show the reticulum of cytoplasm, together with nuclear spindles and asters, stained red, and connective tissue strongly brought out. It may be advisable to acidify the staining bath *very slightly*. Successful stains are admirably sharp: but it is not always easy to obtain them.

304. Orange G.—This is the benzenazo-beta-naphthol-disulphonate of soda (to be obtained from Grübler & Hollborn, and not to be confounded with about a dozen other colours that are on the market under the name of "Orange," with or without a suffix). As indicated by its chemical description, this is an "acid" colour.

It is easily soluble in water, less so in alcohol. Use as directed for Säurefuchsin. Almost, if not quite, as precise a stain as Säurefuchsin. It does not overstain, but may wash out other dyes.

305. Säurefuchsin and Orange G.—I have had good results by mixing the aqueous solutions of these two dyes, but unfortunately have not noted the proportions.

306. Ehrlich-Biondi **Mixture** (or Ehrlich-Biondi-Heidenhain **Mixture**) (*Pflüger's Arch.*, xliii, 1888, p. 40).

To 100 c.c. saturated aqueous solution of orange add with continual agitation 20 c.c. saturated aqueous solution of Säurefuchsin (Acid Fuchsin) and 50 c.c. of a like solution of methyl green.

(According to Krause [*Arch. mik. Anat.*, xlii, 1893, p. 59], 100 parts of water will dissolve about 20 of Säurefuchsin [Rubin S], 8 of orange G, and 8 of methyl green). The solutions must be *absolutely saturated*, which only happens after several days.

Dilute the mixture with 60 to 100 volumes of water. The dilute solution ought to redden if acetic acid be added to it; and if a drop be placed on blotting-paper it should form a spot bluish green in the centre, orange at the periphery. If the orange zone is surrounded by a broader red zone, the mixture contains too much fuchsin.

According to M. Heidenhain's instructions ("Ueber Kern u. Protoplasma," in *Festschr. f. Kölliker*, 1892, p. 115) the orange to be used should be "Orange G;" the Acid Fuchsin or Säurefuchsin should be "Rubin S") ("Rubin" is a synonym of Fuchsin) and the methyl green should be "Methylgrün OO." And it is *absolutely necessary* that these ingredients be those prepared under those names by the *Actienfabrik für Anilinfabrikation in Berlin.* They can be obtained from Grübler & Hollborn, either separately, or as a mixture of the three dyes in powder (which I do not recommend).

The strong solutions directed to be taken readily precipitate on being mixed. To avoid this it is recommended by Squire (*Methods and Formulæ*, etc., p. 37) to dilute them before mixing.

Other proportions for the mixture have been recommended by Krause (*loc. cit. supra*), viz. 4 c.c. of the Säurefuchsin solution, 7 of the orange G, and 8 of the methyl green; the mixture to be diluted 50 to 100-fold with water. THOMÉ (*Arch. mik. Anat.*, lii, 1898, p. 820) gives the proportions 2 : 5 : 8, and dilutes 100-fold.

Stain sections (N.B. *sections only*) for six to twenty-four hours. Dehydrate with alcohol, clear with xylol, and mount in xylol balsam.

In the intention of the observers who have elaborated this stain, it is a *progressive* stain, and *not* a regressive one. It does not require any differentiation, and the sections should be got through the alcohol into xylol as quickly as possible in order to avoid any extraction of the methyl green, which easily comes away in the alcohol.

The best results are obtained with *sublimate material;* chrom-osmium material, and the like, give a much inferior stain. Preparations made with the usual mixture, as given above, are liable to fade; by *acidifying* the mixture a stronger and more sharply selective stain is obtained, which does not fade. But too much acid must not be added, as this would cause a staining of the interfilar substances. According to the *Encycl. mik. Technik,* you may add 15 to 24 drops of 0·2 per cent. acetic acid to 100 c.c. of the diluted solution.

Another process of acidification is given by M. HEIDENHAIN (*Ueber Kern und Protoplasma*, p. 116; for this see *fourth edition.* See also ISRAEL (*Praktikum Path. Hist.*, 2 Aufl., Berlin, 1893, p. 69); TRAMBUSTI (*Ricerche Lab. Anat. Roma*, v, 1896, p. 82: *Zeit. wiss. Mik.*, xiii, 1896, p. 357); and THOMÉ (*op. cit. supra*).

After acidification the solution must not be filtered, and if it has been kept for some time a little more acid must be added.

Before staining (M. HEIDENHAIN, *loc. cit.*), sections should be treated for a couple of hours with 0·1 per cent. acetic acid, then for ten to fifteen minutes with officinal tincture of iodine, and be rinsed with alcohol before bringing into the stain, in which they should remain for twelve to eighteen hours. The treatment with acid is necessary in order to ensure having the sections *acid* on mounting in balsam. The primary object of the iodine is to remove any sublimate from the preparations (Heidenhain's descriptions refer to sublimate objects), but it also is said to enhance the power of staining of the chromatin with methyl green, and to produce a more selective staining of protoplasmic elements.

The stain is a very fine one when successful. But it is *very capricious*. The correct result should be a precise chromatin stain combined with a precise stain of the plastin element or reticulum of cytoplasm by the Säurefuchsin. Now the least defect or excess of acidity causes the plasma stain of the Säurefuchsin to become a diffuse one, instead of being sharply limited to the plastin element. It is difficult to dehydrate the sections without losing the methyl green. For this reason the stain will only work with very thin sections: to be quite sure of good results, the sections should be of not more than 3 μ in thickness, and if they are over five the desired results are almost hopeless. The stain keeps very badly. I admit that the method has its *raison d'être* for the very special objects for which it was imagined—for the researches on cell-granulations for which EHRLICH employed the three colours, or for the researches on the plastin reticulum of cytoplasm for which MARTIN HEIDENHAIN employed the mixture; for the study of gland cells; and for similar objects. But to recommend it, as has been done, as a general stain for ordinary work, is nothing but mischievous exaggeration. Far from having the qualities that should be possessed by a normal section stain, the Ehrlich-Biondi mixture is highly unfitted for ordinary work. Workers have at length found this out, and, after a period of well-nigh unparalleled popularity, this stain is now but little used except for the special purposes above indicated.

307. EHRLICH'S **"Triacid" Mixture.**—This name would seem to indicate that the mixture contains three "acid" colours, which is not the case, methyl green being a strongly "basic" colour. Ehrlich explains in a letter to Mayer (see also EHRLICH and LAZARUS, *Die Anæmie*, 1898, p. 26) that it is so called "because in it all the three basic groups of the methyl green are combined with the acid dye-stuffs." A very pretty conundrum!

The latest receipt (*op. cit.*, p. 28) is as follows:

Prepare separately saturated solutions of orange G, Säurefuchsin, and methyl green, and let them clarify by settling. Then mix, in the order given, using the same measure-glass, 13 to 14 c.c. of the orange, 6 to 7 of the Säurefuchsin, 15 of distilled water, 15 of alcohol, 12½ of the methyl green, 10 of alcohol, and 10 of glycerin. After adding the methyl green, shake well, but do not filter.

The mixture keeps well. I find its qualities and defects to be much those of the Ehrlich-Biondi mixture. The stain seems more powerful but less delicate, and the methyl green in it appears to have more resistance to alcohol, so that it is perhaps better adapted for ordinary work.

Mayer (*Grundzüge*, 1901, p. 212) has simplified the formula thus: Take 1 g. methyl green, 2 g. orange, 3 g. Säurefuchsin, and dissolve in a mixture of 45 c.c. water, 10 c.c. glycerin, and 20 c.c. alcohol of 90 per cent.

Morel and Doleris (*C. R. Soc. Biol.*, liv, 1902, p. 1255) mix 1 vol. of the solution with one of 8 per cent. formalin, and add 0·1 per cent. of acetic acid, and state that thus the methyl green is better fixed in the nuclei.

308. Picric Acid.—Picric acid gives useful plasma stains after carmine and hæmatoxylin. The *modus operandi* is as simple as possible; it consists merely in adding picric acid to the alcohols employed for dehydrating the objects.

Picric acid has considerable power of washing out other anilin stains; and *in combination with hydrochloric acid it very greatly enhances* the power with which this acid washes out carmine stains. It should, therefore, not be added to the acidulated alcohol taken for differentiating borax-carmine stains, or the like, but only to the neutral alcohol used afterwards. It has the great quality that it can be used for staining *entire objects.* And as it is extremely penetrating, it is very much indicated for the preparation of such objects as small Arthropods or Nematodes, mounted whole.

It can in some cases be employed by dissolving it in the solution of another dye (see Picro-carmine, Legal's alum-carmine, § 234, etc.); or (for sections) by dissolving it in the xylol or chloroform used for clearing.

Though picric acid is a useful ground stain, it is at most a rough one, being *very diffuse.* It stains, however, horn, chitin, and muscle, with special energy.

309. Van Gieson's **Picro-Säurefuchsin** (from *Zeit. wiss. Mik.*, xiii, 3, 1896, p. 344).—To a saturated aqueous solution of picric acid is added a few drops of saturated aqueous solution of Säurefuchsin, until the mixture has become garnet-red.

After staining (sections only), rinse with water, dehydrate, and clear in oil of origanum.

Ohlmacher (*Journ. Exper. Med.*, ii, 1897, p. 675) adds 0·5 per cent. of Säurefuchsin to a saturated solution of picric acid which has been diluted with an equal quantity of water. He uses this after previous staining with gentian violet.

Ramón y Cajal recommends 0·1 grm. of Säurefuchsin to 100 of saturated solution of picric acid (Schaffer, *Zeit. wiss. Zool.*, lxvi, 1899, p. 236).

Hansen (*Anat. Anz.*, xv, 1898, p. 152) adds 5 c.c. of 2 per cent. solution of Säurefuchsin to 100 c.c. saturated solution of picric acid, and for staining adds to 3 c.c. of the mixture one third of a drop of 2 per cent. acetic acid, stains for a few minutes or hours, rinses in 3 c.c. of water with 2 drops of the acidified stain added, dehydrates, clears with xylol, and mounts in xylol-balsam. Connective tissue red, elastin and all other elements yellow.

Weigert (*Zeit. wiss. Mik.*, 1904, p. 3) adds 10 parts of 1 per cent. Säurefuchsin to 100 of saturated picric acid.

See also Möller, *op. cit.*, xv, 1898, p. 172.

This stain is generally used as a contrast stain to follow hæmatoxylin (see § 407). It is much recommended by some workers for differentiating connective and elastic tissue and smooth muscle. As a cytological stain I consider it detestable.

310. Flemming's Orange Method (*Arch. mik. Anat.*, xxxvii, 1891, pp. 249 and 685).—Stain (for as much as two or three days, or even weeks, if you want a very powerful stain) in strong alcoholic safranin solution diluted with anilin water (§ 298); rinse in distilled water; differentiate in absolute alcohol, containing at most 0·1 per cent. of hydrochloric acid, until hardly any more colour comes away; stain for one to three hours in gentian violet (§ 299); wash for a short time in distilled water; treat with concentrated, or at least fairly strong, aqueous solution of orange G, which, in virtue of its acid properties, washes out most of the gentian. After at most a few minutes, whilst pale violet clouds are still being given off from the sections on agitation, bring them into absolute alcohol until hardly any more colour comes away, clear in clove or bergamot oil, and mount in damar or balsam before the last pale clouds of colour have ceased to come away. The orange *must* be *orange G* (§ 304).

This is not a triple stain in the sense of giving three different colours in the result; it is a nuclear and plasmatic stain in mixed tones; the orange, apparently, combines with the gentian to form a "neutral" dye, soluble in excess of the orange (§ 216) which thus differentiates the stain.

See also Flemming in *Arch. Anat. Phys., Anat. Abth.*, 1897, p. 175. Never popular, this clumsy and uncertain process is now little used.

311. Reinke's Modification of Flemming's Orange Method (*Arch. mik. Anat.*, xliv, 2, 1894, p. 262).—To a concentrated aqueous solution of gentian violet are added "a few drops" of a like solution of orange G. The solution precipitates in part, owing to the formation of an imperfectly soluble "neutral" colour, but becomes almost clear again if an excess of water be added. A drop of the mixture placed on blotting-paper should form a violet or brown spot with a narrow orange border. The solution is not to be filtered, but the sections are to be stained in the mixture made almost clear by addition of water. It is said that the "neutral" solution may be preserved for future use by adding to it one third of alcohol. After staining (sections, previously stained with safranin), you differentiate rapidly with alcohol and clear with clove oil.

I have tried this process and obtained exactly the same results as with Flemming's process, and so have other workers.

312. Bordeaux R.—An "acid" dye, giving a general stain taking effect both on chromatin and cytoplasm, and, I consider, a very good plasma stain. I use for chrom-osmium material a 1 per cent. solution, and stain for twelve to twenty-four hours. The stain is sufficiently fast.

313. Bordeaux R, Thionin, and Methyl Green (Gräberg, *Zeit. wiss. Mik.*, xiii, 4, 1896, p. 460).

314. Congo Red (Congoroth) (see Griesbach, in *Zeit. wiss. Mik.*, iii, 1866, p. 379).—An "acid" colour. Its solution becomes blue in presence of the least trace of free acid (hence Congo is a valuable reagent for demonstrating the presence of free acid in tissues; see the papers quoted *loc. cit.*). A stain much of the same nature as Säurefuchsin. It is useful for staining some objects during life (see *ante*, § 221). Carnoy (*La Cellule*, xii, 2, 1897, p. 216) has had very good results with it as a secondary stain employed after hæmatoxylin of Delafield. He used a 0·5 per cent. solution in water. Note that this colour is not to be confounded with other Congos, as Congo yellow, or brilliant Congo. It is one of the azo dyes.

Loisel (*Journ. de l'Anat. et de la Phys.*, 1898, p. 230) says, concerning the reaction with free acids, that the colour is azure-blue with mineral acids and dark violet with organic acids. In the presence of chlorine compounds it gives the same reaction as with acids. In the presence of ammoniacal liquids it will not give the reaction with CO_2 nor with acetic or lactic acid. See WURSTER, *Centralb. Physiol.*, 1887, p. 240.

315. Congo-Corinth. Also an acid dye, of the Amido-azo group. HEIDENHAIN (*Zeit. wiss. Mik.*, xx, 1903, p. 179) recommends Congo-Corinth G (or the allied colour **Benzopurpurin 6 B** (Elberfelder Farbwerke). Sections *must be made alkaline* before staining. This may be done by treating them with very weak sal ammoniac or caustic soda, in alcohol. After staining, pass through absolute alcohol into xylol. Used after alum hæmatoxylin, the stain of which it does not cause to fade.

316. Benzopurpurin.—According to GRIESBACH (*loc. cit.*, § 314), another "acid" colour very similar in its results to Congo red. It has been commended as a plasma stain. See also ZSCHOKKE (*ibid.*, v. 1888, p. 466), who recommends **Benzopurpurin B,** and says that weak aqueous solutions should be used for staining, which is effected in a few minutes, and alcohol for washing out. **Deltapurpurin** has, it is said, similar properties, and may be used in the same way.

See last § as to the necessity of *alkalising* the sections, which Heidenhain states is necessary with all dyes of this group.

317. Blauschwarz B and **Brillantschwarz 3 B** (HEIDENHAIN, *op. cit.*, § 315, p. 183). Acid azo dyes. To be used in 1 per cent. solution with sections of sublimate material, staining for five to ten minutes. Then stain in a basic dye, such as toluidin blue or safranin, which forms a neutral dye that may be differentiated as explained for Thiazin red or Coerulein, § 328.

318. Neutral Red (Neutralroth) (EHRLICH, *Allg. med. Zeit.*, 1894, pp. 2, 20; *Zeit. wiss. Mik.*, xi, 1894, p. 250; GALEOTTI, *ibid.*, p. 193).—A "basic" dye. The term "neutral" refers to the hue of its solution. Its neutral red tint is turned bright red by acids, yellow by alkalies. The stain in tissues is in general metachromatic, nuclei being red, cell-bodies yellow (*cf.* ROSIN, in *Deutsche med. Wochenschr.*, xxiv, 1898, p. 615; *Zeit. wiss. Mik.*, xvi, 2, 1899, 238). Up to the present this colour has chiefly been employed for *intra-vitam* staining. Tadpoles kept for a day or two in a solution of 1 : 10,000 or 100,000 absorb so considerable a quantity of the colour that all their tissues appear of a dark red. The stain is limited to cytoplasmic granules (EHRLICH), and to the contents of mucus cells (GALEOTTI).

According to a further study of this colour by Ehrlich and Lazarus (*Spec. Pathol. und Therapie,* herausgeg. von Nothnagel, viii, 1, 1898, p. 1; *Zeit. f. wiss. Mik.,* xv, 3, 1899, p. 338) it may be used for *intra-vitam* staining of tissues in the same way as methylen blue, by injection or immersion with contact of air. It is especially a *granule* stain. Similar results are recorded by Arnold (*Anat. Anz.,* xvi, 1899, p. 568, and xxi, 1902, p. 418). See also Loisel (*Journ. de l'Anat. et de la Physiol.,* 1898, pp. 197, 210, 217) (*intra-vitam* staining of sponges); and Prowazek, *Zeit. wiss. Zool.,* lxii, 1897, p. 187 (*intra-vitam* staining of Protozoa). I myself have had very good results with it as an *intra-vitam* stain.

According to Golovine (*Zeit. wiss. Mik.,* xix, 1902, p. 176) the stain may be fixed in the tissues by means of sublimate, chromic acid, bichromates, picric acid, or platinum chloride, followed by molybdate of ammonium of different strengths, from a solution containing 70 c.c. of water, 30 of 90 per cent. alcohol, and 40 drops of saturated sol. of the molybdate in water to one containing 10 c.c. of water, 90 of alcohol, and twenty drops of the molybdate, with dehydration in saturated solution of picrate of ammonia in absolute alcohol.

It has also been found useful for staining, in hardened material, the corpuscles of Nissl (*q. v.*) in nerve-cells. S. Mayer (*Lotos,* Prague, 1896, No. 2) states that it also stains degenerating myelin. The solutions that have been employed for staining fixed material are strong aqueous ones, 1 per cent. to concentrated.

319. The Eosins, found in commerce under the names of **Eosin, Saffrosin, Primerose Soluble, Phloxin, Bengal Rose, Erythrosin, Pyrosin B, Rose B, a l'Eau,** etc., are all "acid" phthaleïn colours. They are not quite identical in their properties, but vary according to the different modes of manufacture. Most of them are soluble both in alcohol and in water, but some only in alcohol ("*Primerose à l'Alcool*").

They are all diffuse stains, formerly much used as contrast stains, less so now.

Their chief use is in combinations or mixtures, to be given further on. For **Bengal Rose** see Griesbach, *Zool. Anz.,* 1883, p. 172.

Eosin is a specific stain for red blood corpuscles, and also for certain granules of leucocytes (see § 719).

The yolk of some ova takes the stain strongly, so that it is useful in some embryological researches.

320. EHRLICH'S **Indulin-Aurantia-Eosin,** or **Acidophilous Mixture,** or **Mixture C,** or **Mixture for Eosinophilous Cells** (from the formula kindly sent me by Dr. GRÜBLER).—Indulin, aurantia, and eosin, of each two parts; glycerin, thirty parts. This gives a very thick, syrupy solution. To use it, cover-glass preparations may be floated on to it; or sections on slides may have a few drops poured on to them, the slide being laid flat till the stain has taken effect (twenty-four hours for Flemming material). This mixture was invented for the purpose of obtaining a specific stain of certain granules of leucocytes. It has been pointed out by NIKIFOROW (*Zeit. wiss. Mik.*, viii, 1891, p. 189; and xi, 1894, p. 246) that it is also available for staining sections. I find this is the case. With Flemming material it gives a powerful and good stain, which is much more resistant to alcohol than that of the EHRLICH-BIONDI mixture, and is therefore much more adapted to ordinary work Chromatin in my preparations is of a very dark blue, cytoplasm being of a lighter blue (except where it has taken the stain of the aurantia or eosin). It will thus be seen that the indulin in this combination behaves in a manner quite opposed to its behaviour when used alone (see § 330). The stain is said to keep well.

ISRAEL (*Praktik. Path. Hist.*, Berlin, 1893, p. 68) gives a more complicated receipt.

321. Methyl Green and Eosin (CALBERLA, *Morph. Jahrb.*, iii, 1877, Heft 3, p. 625; LIST, *Zeit. wiss. Mik.*, ii, 1885, p. 147; BALBIANI, *Ann. Microgr.*, Paris, vii, 1895, p. 245; RHUMBLER, *Zeit. wiss. Zool.*, lxi, 1895, p. 38).—See early editions.

322. Methylen Blue and Eosin (CHENZINSKY, quoted from *Zeit. wiss. Mik.*, xi, 2, 1894, p. 269).

Methylen blue, sol. sat. in water	40
Eosin, 0·5 per cent. in 70 per cent. alcohol	20
Distilled water, or glycerin	40

This solution will only keep for about eight days.

It has been recommended as a specific stain for blood.

The mixture of PIANESE (*ibid.*, xi, 1894, p. 345) contains the same ingredients in the same proportions, with the addition of a considerable proportion of carbonate of lithia.

See also the mixture of BREMER (*Arch. mik. Anat.*, xlv, 1895, p. 446). I have tried CHENZINSKY'S mixture as a tissue stain, without good results; but see ROSIN, *Berliner klin. Wochenschr.*, 1898, p. 251; *Zeit. wiss. Mik.*, xvi, 1899, p. 223, and xvii, 1900, p. 333.

LAURENT (*Centralb. allg. Path.*, xi, 1900, p. 86; *Zeit. wiss. Mik.*, xvii, 1900, p. 201) explains that the effects of this mixture are due to the formation of a "neutral" dye, for the preparation of which he gives complicated instructions. It is on sale by Grübler and Hollborn.

See also for similar mixtures § 719.

323. Light Green (Lichtgrün S. F.).—An "acid" colour, soluble in water or alcohol, and a good plasma stain.

BENDA (*Verh. physiol. Ges. Berlin*, Dec. 18th, 1891, Nos. 4 u. 5) stains sections for twenty-four hours in anilin-water safranin solution, then for about half a minute in a solution of 0·5 grm. Lichtgrün or **Säureviolett** (Grübler) in 200 c.c. of alcohol, dehydrates and mounts in balsam. This process gives one of the most beautiful stains known to me. It requires very thin sections and there is always risk of the safranin being washed out. The Lichtgrün stain unfortunately does not keep at all well.

324. Janus Green (MICHAELIS, *Arch. mik. Anat.*, lv, 1900, p. 565). Used in solution of 1 : 30,000 for staining certain granules (pancreas, salivary glands, etc.) in the fresh state.

325. Malachite Green (syn. **Solid Green, Victoria Green, New Green, Benzoyl Green, Fast Green**).—A basic colour, which has been used as a plasma stain for the ova of *Ascaris* by VAN BENEDEN and NEYT. These authors used it for glycerin preparations. See also § 398.

326. Iodine Green ("HOFMANN'S **Grün**"), see GRIESBACH (*Zool. Anz.*, No. 117, vol. v, 1882, p. 406).—The colour is now no longer manufactured for industrial purposes, but may be obtained from C. A. F. Kahlbaum's Chemische Fabrik, Berlin, S.C. (*Zool. Anz.*, No. 130, 1883, p. 56). Stain essentially that of methyl green, but plasma often violet through the presence of a violet impurity (MAYER, *Mitth. Zool. Stat. Neapel*, xii, 1896, p. 311; see also earlier editions). It is now only used by botanists.

327. Thiophen Green (Thiophengrün), see KRAUSE, *Intern. Monatsschr. Anat., etc.*, iv, 1887, Heft 2.

328. Coerulein S., a green "acid" dye, is recommended for the staining of muscle-fibrils by M. v. LENHOSSÉK (*Anat. Anz.*, xvi, 1899, p. 339).

Heidenhain (*ibid.*, xx, 1901, p. 36) produces a "neutral" dye in the tissues (§ 302) by first staining strongly in a saturated solution of coerulein S, then for one to twelve hours in a basic dye, such as safranin or toluidin blue (1 : 1000 in water) or methylen blue (0·5 : 1000 in water). All the solutions should be lightly acidified, and after staining sections should be differentiated with absolute alcohol, or if this does not suffice, with methyl alcohol.

He proceeds similarly with **thiazin brown, thiazin red,** or other acid dyes for the primary stain. For the study of muscle and connective tissue.

329. Quinoleïn Blue (Cyanin, Chinolinblau; v. Ranvier, *Traité*, p. 102).—Quinoleïn is said by Ranvier to have the property of staining fatty matters an intense blue.

It is useful for staining Infusoria, which in dilute solution it stains during life. See the methods of Certes.

From the reactions mentioned by Ranvier it would seem that his "bleu de quinoléine" is not the preparation that usually goes under that name. See Ehrlich, in *Arch. mik. Anat.*, xiii, 1877, p. 266.

330. Indulin and Nigrosin.—The **Indulins** are a group of dyes related to the base violanilin. They may be either "basic" colours or "acid." The soluble indulins of commerce are generally "acid." (Benedikt and Knecht, *Chemistry of Coal-tar Colours*, p. 187). They occur under the brands (not strictly synonyms) of **Indulin, Nigrosin, Indigen, Coupier's Blue, Fast Blue R, Fast Blue B, Blackley Blue, Guernsey Blue, Indigo substitute.** According to Behrens the name Indulin is generally given to a bluish brand, and that of Nigrosin to a blacker one.

Nigrosin, used with sublimate material, I find stains both nuclei and cytoplasm, the chromatin strongly. I do not consider it a very good plasma stain. It will not give the stain at all with chrom-osmium material.

According to Calberla (*Morph. Jahrb.*, iii, 1877, p. 627) the concentrated aqueous solution of *Indulin* should be diluted with six volumes of water. Sections will stain in the dilute solution in five to twenty minutes; they may be washed in water or in alcohol, and examined either in glycerin or oil of cloves.

According to Calberla it never stains nuclei; the remaining cell-contents and intercellular substance are stained blue. This seems to me to be, roughly, correct.

331. Safranin and Nigrosin (or Indigo-Carmine) (Kossinski, *Zeit. wiss. Mik.*, vi, 1880, p. 61).—See early editions.

332. Picro-Nigrosin, Pfitzer (*Deutsch Botan. Gesellsch.*, 1883, p. 44) dissolves nigrosin in a saturated solution of picric acid in water, and uses it for fixing and staining at the same time, on the slide. See also § 687.

333. Anilin Blue.—Under this title are comprised various "basic" derivatives of the base rosanilin. They occur under the names **Spirit Soluble Blue** (**Bleu Alcool**), **Gentian Blue 6 B**, **Spirit Blue O**, **Opal Blue**, **Bleu de Nuit**, **Bleu Lumière**, **Parma Blue**, **Bleu de Lyon**. Formulæ of the older authors for staining with "Anilin blue" should, I think, be disregarded, as it would probably now be impossible to ascertain what colour was meant by them.

The only one of the above-mentioned colours of which I have any personal knowledge, or that appears really valuable, is **Bleu de Lyon**. (Some authors gives the names Bleu de Nuit and Gründstichblau as synonyms of Bleu de Lyon. The *Encycl. mik. Technik.* says it is "Anilinblau B— 6 B," with many synonyms, or designations of brands, Parma blue being "Anilinblau R or 2 R"). I find it a fairly good stain, giving very good differentiations of nerve tissue, and of cartilage (as has already been pointed out by BAUMGARTEN and by JACOBY). The older mode of using it (MAURICE and SCHULGIN) was to stain in bulk with it after borax-carmine, using a very dilute alcoholic solution. BAUMGARTEN and JACOBY stain sections in a 0·2 per cent. alcoholic solution. I have experimented with safranin as the chromatin stain, and do not think the combination can be recommended. For if you stain first with the blue, the safranin will extract it if allowed to act for more than a short time. And if you stain first with the safranin, the blue will extract it very quickly. But it works well with carmine.

TONKOFF (*Arch. mik. Anat.*, lvi, 1900, p. 394) adds a little tincture of iodine to the solution of the dye, or mordants the sections with iodine.

334. Carmine Blue (**Bleu Carmin Aqueux**), another "basic" anilin dye, prepared by Meister, Lucius, and Brunig, at Höchst-a-M.).—JANSSENS (*La Cellule*, ix, 1893, p. 9) states that this colour possesses a special affinity for the parts of cytoplasm that are undergoing cuticular differentiation. He uses it in alcoholic solution acidified. The stain will bear mounting in balsam.

335. Methyl Blue.—Under this title are comprised some other derivatives of the base rosanilin. They are "acid" colours. Here belong **Methyl Blue**, **Cotton Blue**, **Water Blue** (**Wasserblau**), **Methyl Water-Blue**, **China Blue** (**Chinablau**), **Soluble Blue**.

Amongst these **Water Blue (Wasserblau)** possesses some useful properties. According to MITROPHANOW (quoted from *Zeit. wiss. Mik.*, v, 1888, p. 513), used in concentrated aqueous solution it gives a very good double stain with safranin. It is very resistant to alcohol. Using the Wasserblau first, and then the safranin, I have had some interesting results, and as the process is easy to carry out I think it may be recommended. The Wasserblau must be used first, as if used after the safranin it will destroy the stain in a short time. With chrom-osmium material, twelve to twenty-four hours in the blue, and four or five in the safranin, may not be too much. My stains have not kept well.

MANN (*op. cit.* xi, 1894, p. 490) has used it with eosin, in a complicated way, for staining ganglion cells.

336. Anilin Blue-black.—A preparation cited under this name has been recommended by BEVAN LEWIS and others for nervous tissue. Unfortunately these authors have not given the chemical description of the colour used by them. The *Encycl. mik. Technik* concludes that it must have been "a water-soluble anilin blue, from Levinstein's at Manchester." Dr. GRÜBLER writes me that the anilin blue-black of his list is the oxyazo colour blue-black B or AZOSCHWARZ; but that dye had not been discovered when Bevan Lewis wrote.

337. Violet B (or Methyl Violet B) (S. MAYER, *Sitzb. k. k. Akad. wiss. Wien*, iii Abth, February, 1882).—Used in solutions of 1 grm. of the colour to 300 grms. of 0·5 per cent. salt solution, and with *fresh tissues* that have not been treated with any reagent whatever, this colour gives a stain so selective of the elements of the vascular system that favourable objects, such as serous membranes, appear as if injected. The preparations do not keep well; acetate of potash is the least unsatisfactory medium for mounting them in, or a mixture of equal parts of glycerin and saturated solution of picrate of ammonia (*Anat. Anz.*, 1892, p. 221). See also § 662.

The allied dye, **Crystal Violet,** has been employed for staining sections, *e.g.* by KROMAYER and others. BENDA (*Neurol. Centralb.*, xix, 1900, p. 792) stains in a mixture of 1 vol. saturated sol. of the dye in 70 per cent. alcohol, 1 vol. 1 per cent. sol. of hydrochloric acid in 70 per cent. alcohol, and 2 vols. of anilin water, the liquid being warmed until vapour is given off, then the sections dried with blotting-paper, treated one minute with 30 per cent. acetic acid, dehydrated with alcohol and cleared with xylol.

338. Kresyl Violet.—An oxyazin dye, giving metachromatic stains HERXHEIMER (*Arch. mik. Anat.*, liii, 1899, p. 519, and liv, p. 289) stains sections of skin with **Kresylecht-violett.** Nuclei blue, plasma reddish. Similarly FICK (*Centralb. allg. Path.*, xiii, 1902, p. 987 ; *Zeit. wiss. Mik.*. xx, 1903, p. 223), staining for three or four minutes in a concentrated aqueous solution, and differentiating in alcohol until the connective tissue has become colourless. Keratohyalin violet-red to salmon-coloured.

339. Säureviolett, see § 323.

340. Benzoazurin may be made to give either a diffuse or a nuclear stain, according to MARTIN (see *Journ. Roy. Mic. Soc.*, 1890, p. 114).

341. Baumgarten's Fuchsin and Methylen Blue (*Zeit. wiss. Mik.*, i, 1884, p. 415).—Stain sections (of chromic objects) for twenty-four hours in a stain made by adding 8 to 10 drops of concentrated alcoholic solution of fuchsin to a watch-glassful of water. Rinse with alcohol, and stain for four or five minutes in concentrated aqueous solution of methylen blue, wash out with alcohol for five to ten minutes, and clear with clove oil. Nuclei red, tissues blue, by *substitution.*

342. RAWITZ' "Inversion" Plasma Stains.—It has been discovered by RAWITZ that by means of appropriate mordants certain basic anilins, which by the usual methods of regressive staining are pure chromatin stains, may be made to afford a pure plasma stain, thus giving an "inversion" of the usual stain. The stain, in my opinion, is a vile one. For details see *fourth edition,* or RAWITZ (*Sitzb. Gesnaturf. Freunde, Berlin,* 1894, p. 174 ; *Zeit. wiss. Mik.*, xi, 1895, p. 503 ; and his *Leitfaden f. hist. Untersuchungen,* Jena, 1895, p. 76).

343. Artificial Alizarin (RAWITZ, *Anat. Anz.*, xi, 10, 1895, p. 294).—RAWITZ has also worked out a process of obtaining a double stain (chromatin and cytoplasm being stained of different colours) by means of artificial Alizarin, or Alizarin-cyanin. The process is an adjective one, requiring the use of special mordants supplied by the colour manufacturers, and is very complicated. See *last edition.*

344. BENDA'S Alizarin Stain for Mitochondria (*Ergebnisse der Anat.*, xii, 1902 (1903), p. 752, previously published in other places). Harden for eight days in strong liquid of Flemming (the acetic acid therein being reduced to three drops). Wash for an hour in water and put for twenty-four hours into a mixture of equal parts of pyroligneous acid and 1 per cent. chromic acid, then for twenty-four hours into bichromate of potash of 1 : 1000, wash for twenty-four hours and imbed in paraffin. Sections on the slide are mordanted for twenty-four hours in a stove with 4 per cent. solution of ferric alum or diluted *liq. ferri sulfur. oxydat.*, then rinsed with water and put for twenty-four hours in the stove into an amber-yellow aqueous solution of Kahlbaum's sulfalizarinate of soda, prepared by dropping an alcoholic solution thereof into water.

Rinse in water, flood the slides with solution of crystal violet, § 337, diluted with an equal vol. of water, and warm till vapour is given off. Rinse, differentiate one or two minutes in 30 per cent. acetic acid, dry, dip into acetone, pass through bergamot oil into xylol and balsam. Mitochondria violet, chromatin and "archoplasm" brown-red. **Benda** supposes that the iron and alizarin form a mordant for the anilin dye. See also §§ 657, 658.

CHAPTER XVI.

METHYLEN BLUE.

345. Methylen Blue is a "basic" dye, being the chloride or the zinc chloride double salt of tetramethylthionin. It appears that some persons have confounded it with the "acid" dye methyl blue, to which it has not, histologically, any resemblance.

Commercial methylen blue sometimes contains as an impurity a small quantity of a red dye, which used to be taken to be methylen red. This impurity is present from the beginning in many brands of methylen blue, is frequently developed in solutions of the dye that have been long kept (so-called "ripened" solutions), and is still more frequently found in kept *alkaline* solutions. It is a reddish-violet dye, and according to NOCHT (*Centralb. Bakteriol.*, xxv, 1899, pp. 764—769; *Zeit. wiss. Mik.*, xvi, 1899, p. 225) it is not methylen red, nor methylen violet either, but a new colour, for which NOCHT proposes the name "Roth aus Methylenblau."

According to MICHAELIS (*Centralb. Bakteriol.*, xxix, 1901, p. 763, and xxx, 1901, p. 626; *Zeit. wiss. Mik.*, xviii, 1902, p. 305, and xix, 1902, p. 68) this dye is "Methylenazur," an oxidation-product of methylen blue. It is an energetic dye, of markedly metachromatic action, and to it are due the metachromatic effects of methylen blue solutions (methylen blue itself is not metachromatic).

The presence of this impurity in methylen blue is not always an undesirable factor; on the contrary, it sometimes affords differentiations of elements of tissues or of cells that cannot be produced by any other means. It is present in the stains of LOEFFLER and of ROMANOWSKY, § 719. Methylen blue that contains it is known as *polychromatic methylen blue*, and is employed for staining *certain cell-granules*. It can be obtained from Grübler & Hollborn. MICHAELIS (*op. cit.*) makes a methylen blue solution containing the "azur" as follows: 2 gr. of medicinal methylen blue are dissolved in 200 c.c. of water, and 10 c.c. of $\frac{1}{10}$ normal solution of caustic soda added. Boil for a quarter of an hour; after cooling add 10 c.c. of normal sulphuric acid, and filter.

There are several sorts of methylen blue sold, the most important being—"methylen blue, according to EHRLICH"; "methylen blue, according to KOCH"; "methylen blue BX, according to S. MAYER"; "Methylenblau, medic. pur."

The colour to be employed for *intra-vitam nerve staining* should be as pure as possible. APÁTHY (*Zeit. wiss. Mik.*, ix, 1893, p. 466) writes that the best—in fact, the only one that will give *exactly* the results described by him—is that of E. MERCK, of Darmstadt, described as "*medicinisches* Methylenblau." DOGIEL (*Encycl. mik. Technik*, p. 811) has had his best results with "Methylenblau n. Ehrlich," or "B X," obtained from Grübler & Hollborn.

346. The Uses of Methylen Blue.—As a histological reagent it is used for sections of hardened central nervous tissue, in which it gives a specific stain of medullated nerves (*post*, Part II). It gives more or less specific stains of the basophilous granulations of "Mastzellen" and plasma-cells, and the granules of NISSL in nerve-cells, also mucin (for all of which see also Part II). It stains a large number of tissues *intra vitam*, with little or no interference with their vital functions. And last, not least, it can be made to furnish stains of nerve tissue, intercellular cement substances, lymph spaces, and the like, that are essentially identical with those furnished by a successful impregnation with gold or silver, and are obtained with greater ease and certainty; with this difference, however, that gold stains a larger number of the nervous elements that are present in a preparation, sometimes the totality of them; whilst methylen blue stains only a selection of them, so bringing them more prominently before the eye, and allowing them to be traced for greater distances. So that in this respect methylen blue behaves more like Golgi's chrome silver impregnation.

347. Staining *in toto* during Life.—Small and permeable aquatic organisms may be stained during life by adding to the water in which they are confined enough methylen blue to give it a very light tint. If transparent organisms be taken, they may be examined alive without further manipulation at any desired moment, and will be found after a time to be partially stained—that is, it will be found that certain tissues have taken up the colour, others remaining colourless. If now you put back the animals into the tinted water and wait, you will find on examination after a sufficient lapse of time that further groups of tissues have become stained. Thus it was found by Ehrlich (*Biol. Centralb.*, vi, 1886, p. 214 ; *Abh. k. Akad. Wiss. Berlin*, February 25, 1885), to whom the principle of this method is due, that on injection of the colour into living animals axis-cylinders of *sensory* nerves stain, whilst *motor* nerves remain colourless. [The motor nerves, however, will also stain, though later than the sensory nerves.] It might be supposed that by continuing the staining for a sufficient time, a point would be arrived at at which *all* the tissues would be found to be stained. This, however, is not the case. It is always found that the stained tissues only keep the colour that they have taken up for a short time after they have attained the maximum degree of coloration of which they are susceptible ; as soon as that point is attained they begin to discharge the colour even more quickly than they took it up. According to Ehrlich this decoloration is explained as follows : methylen blue, on contact with reducing agents in alkaline solution can be reduced to a colourless body, its " leucobase." Now living or recently dead tissue elements are, or may be, both alkaline and very greedy of oxygen, and thus act on the dye as reducing agents. The leucobase thus formed is easily reoxidised into methylen blue by oxidising substances, or acids, or even by the mere contact of air—which latter property is taken advantage of in practice.

It follows that a total stain of all the tissues of a living intact organism can hardly be obtained under these conditions, but that a specific stain of one group or another of elements may be obtained in one of two ways. If the tissue to be studied be one that stains earlier than the others, it may be studied during life at the period at which it alone has attained the desired intensity of coloration. If it be one that stains later than the others, it may be studied at the period at which the earlier stained elements have already passed their point of maximum coloration and have become sufficiently decoloured, the later stained ones being at a point of desired intensity. Or the observer may fix the stain in either of these stages and preserve it for leisurely study by means of one of the processes given § 351.

The proper strength of the very dilute solutions to be employed for the staining of living organisms must be made out by experiment for each object. I think the tint is practically a sufficient guide, but it may be stated that when in doubt a strength of 1 : 100,000 may be taken, and increased or diminished as occasion may seem to require. Zoja (*Rendic R. Ist. Lombardo*, xxv, 1892 ; *Zeit. wiss. Mik.*, ix, 1892, p. 208) finds that for Hydra the right strength is from 1 : 20,000 to 1 : 10,000.

The stain is capricious. It is not possible to predict without trial which tissues will stain first in any organism. It is to be remarked that the stain penetrates very badly, which is no doubt one cause of its capriciousness. Gland cells generally stain early ; then, in no definable order, other epithelium cells, fat cells, plasma cells, "Mastzellen," blood and lymph corpuscles, elastic fibres, smooth muscle, striated muscle. There are other elements that stain in the living state, but *not* when the staining is performed by *simple immersion of intact animals* in a dilute staining solution in the manner we are considering. Chief amongst these are *nerve-fibres* and *ganglion cells, which remain unstained in the intact organism*—most likely, so far as I can see, for the simple reason that the stain is not able to penetrate them. To get these stained, it is necessary to isolate them sufficiently, as explained in the following sections.

348. Staining Nervous Tissue during Life.—It was made out by Ehrlich (*op. cit.*, last §) that by injecting a solution of methylen blue into the vessels or tissues of living animals and shortly afterwards cutting out and examining small pieces of their tissues, these will be found to be intensely stained in some of their elements (chiefly nervous). If the tissues are mounted under a cover-glass, the stain will fade in a short time ; but if the cover-glass be removed, so that oxygen can have access to the tissues, the stain will be restored, as explained last §. The chief elements stained in this way are peripheral nerves, and amongst these more especially axis-cylinders of sensory nerves.

Ehrlich held, and it has long been held by observers, that the stain so obtained is a product of a *vital* reaction of the tissues, and that it cannot be obtained with dead material. DOGIEL, however (*Arch. mik. Anat.*, xxxv, 1890, pp. 305 *et seq.*), found that muscle nerves of limbs of the frog could be stained as much as from three to eight days after the limbs had been removed from the animal. He concludes, indeed, that the reaction shows that the nerves were still living at that time. But it seems more natural to conclude with APÁTHY (*Zeit. wiss. Mik.*, ix, 1892, pp. 15 *et seq.*) that nerve-tissue can be stained after life has ceased. APÁTHY has directly experimented on this point, and sums up the necessary conditions as follows: The tissue need not be living, but must be fresh; nothing must have been extracted from it chemically, and its natural state must not have been essentially changed by physical means. For example, the tissue must not have been treated with even dilute glycerin, nor with alcohol, though a treatment for a short time with physiological salt solution is not very hurtful; it must not have been coagulated by heat.

But be this as it may, all observers are agreed that to obtain the *best* stains of nerves, the tissues must be quite fresh, either removed during the life of the animal or at most an hour or two after death.

As above explained, the primary stain obtained by injecting methylen blue, or immersing tissues in it, only lasts a very short time. In order to get it to last long enough for study, it must be re-blued by oxidation (see last §). It is therefore the usual practice to dissect out the tissues to be examined, and leave them for some time exposed to the air. This is done in order that they may take up from the air the necessary *oxygen*. Another consideration that justifies the practice is that by exposure to air the preparations take up a trace of *ammonia*, and APÁTHY has experimentally established that this is an important factor in the sharpness of the stain. Ehrlich also (*op. cit.*) holds that an alkaline reaction of the tissues is a necessary condition to the stain. Apáthy further holds that the stain is a *regressive* one, easily washed out by the surrounding liquid; and in order to prevent this washing-out being excessively rapid, it is desirable to have it go on in presence of as little liquid as possible.

349. The Modes of Staining.—The practice of the earlier workers at this subject was (following Ehrlich) to *inject* methylen blue into the vascular system or body-cavity of a living animal, wait a sufficient time for it to take effect on the organ to be stained, then remove the organ for further preparation and study. And there appears to have been a belief with some workers that it was an essential or at least a desirable condition to the production of the stain that it should have been brought about by injection of the colouring matter into the entire animal. It is now known that this is generally immaterial, and that the reaction can equally well be obtained by removing the organ and subjecting it to *a bath* of the colouring matter in the usual way. But it would also appear that in some cases the procedure by injection is preferable, if not necessary. Another procedure, the *diffusion* process, which gives good results in special cases, is by *dusting* the tissues with the solid dye in powder.

350. The Solutions employed.—The solutions used *for injection* are generally made in salt solution (physiological, or a little weaker); those for staining by immersion, either in salt solution or other "indifferent" liquid, or in pure water. Very various strengths have been employed. The earlier workers generally took concentrated solutions. Thus Arnstein (*Anat. Anz.*, 1887, p. 125) injected 1 c.c. of saturated (*i. e.* about 4 per cent.) solution into the vena cutanea magna of frogs, and removed the organ to be investigated after the lapse of an hour. Biedermann (*Sitzb. Akad. Wiss. Wien, Math. Nat. Cl.*, 1888, p. 8) injected 0·5 to 1 c.c. of a nearly saturated solution in 0·6 per cent. salt solution into the thorax of crayfishes, and left the animals for from two to four hours before killing them. S. Mayer (*Zeit. wiss. Mik.*, vi, 1889, p. 423) took a strength of 1 : 300 or 400 of 0·5 per cent. salt solution. This can be introduced into the system either by means of a syringe or other injecting apparatus, or by auto-injection through the heart. Even rabbits support this operation if artificial respiration be maintained. The solutions of Retzius are of the same strength. But the tendency of more recent practice is decidedly towards the employment of weaker solutions. Apáthy (*ibid.*, ix, 1892, pp. 25, 26 *et seq.*) finds that it is not only superfluous, but positively disadvantageous, to take solutions stronger than 1 : 1000. Dogiel also (*Encycl. Mik. Technik*, p. 815) holds that such strong solutions as 1 to 4 per cent. are disadvantageous, as they produce a staining of other elements besides the nerves. He recommends $\frac{1}{8}$ to $\frac{1}{4}$ per cent, or at most $\frac{1}{2}$ per cent. For warm-blooded animals the solution should be warmed to 36° or 37° C., and before sending in the injection the blood-vessels should be well washed out with similarly warmed salt solution. The injected organs may be removed after twenty to thirty minutes. They should be placed on a thin layer of spun glass moistened with weak ($\frac{1}{8}$ to $\frac{1}{15}$ per cent.) methylen blue, or simply spread out on a slide, and the whole placed in a Petri dish with a layer of the methylen blue on the bottom. The dish is best placed in a stove at 36° C., and after 15 to 30 minutes (if the pieces are thin) or 1 hour to 1$\frac{1}{2}$ hour (if they are thick) specimens may be removed for examination or preservation. Or, without using the stove, specimens may be removed 10 to 20 minutes after injection, placed on a slide, and moistened with weak methylen blue or salt solution, and brought under the microscope. Then as soon as the stain is sufficiently brought out (40 to 60 minutes) they may be fixed (§ 351).

For staining *by immersion* similar solutions to those used for injecting may be employed, but they should, if anything, be still weaker. Dogiel (*Arch. mik. Anat.*, xxxv, 1890, p. 305) places objects in a few drops of aqueous or vitreous humour, to which are added two or three drops of a $\frac{1}{16}$ to $\frac{1}{15}$ per cent. solution of methylen blue in physiological (0·75 per cent.) salt solution, and exposes them therein to the air. In thin pieces of tissues the stain begins to take effect in five or ten minutes, and attains its maximum in from fifteen to twenty minutes. For thicker specimens—retina, for instance—several hours may be necessary, the preparation being kept just moist by occasional treatment with a drop or two of indifferent liquid or methylen blue solution, added by turns. The reaction is quickened by putting the preparations into a stove kept at 30° to 35° C. Rouget (*Compt. Rend.*, 1893, p. 802) employed a 00·5 per cent. solution in 0·6 per cent. salt solution (for muscles of Batrachia). Allen (*Quart. Journ. Mic. Sci.*, 1894, pp. 461, 483) takes for embryos of the lobster a solution of 0·1 per cent. in 0·75 per cent. salt solution, and dilutes it with 15 to 20 volumes of sea water. Seidenmann (*Zeit. wiss. Mik.*, xvii, 1900, p. 239) takes for the choroid a solution of 0·02 per cent. in 0·5 per cent. salt solution.

Apáthy (*Zeit. wiss. Mik.*, ix, 1892, p. 15 : see also his *Mikrotechnik*, p. 172) proceeds as follows for *Hirudinea* and other Invertebrates. A portion of the ventral cord is exposed, and if it be considered desirable dissected out, but the sinus and pigmented connective tissue around it had better not be removed till the staining and fixation are completed. If, however, it be desired to stain as many ganglion cells as possible, as well as fibres, the lateral nerves, as well as the connectives, should be cut through near a ganglion. The preparation is then treated with the stain. This is, for the demonstration chiefly of fibres in *Hirudo* and *Pontobdella*, either a 1 : 1000 solution in 0·5 to 0·75 per cent. salt solution, allowed to act for ten minutes; or a 1 : 10,000 solution allowed to act for an hour to an hour and a half; or a 1 : 100,000 solution allowed to act for three hours (*Lumbricus* requires twice these times; *Astacus* and *Unio* require three times; medullated nerves of Vertebrates four times). For the demonstration of ganglion cells the stain is allowed to act three or four times as long.

The staining having been accomplished, the preparations from the 1 : 1000 solution are washed in salt solution for an hour; those from the 1 : 10,000 solution for a quarter of an hour; those from the 1 : 100,000 solution need not be washed at all. They are then treated with one of the ammoniacal fixing and differentiating liquids described in § 351. This is done by pouring the liquid over them, and leaving them in it *without moving them about in it* for at least an hour, and by preference in the dark. The further treatment is as described in § 351.

The object of the ammonia in these liquids is to *differentiate* the stain—to produce an artificial "secondary differentiation." It acts by washing out the absorbed colour from certain elements, others resisting its action longer, much as HCl alcohol washes out a borax-carmine stain. The *diffusion* method, due to Ramón y Cajal (*Rev. trim. Microgr.*, i, 1896, p. 123; *Zeit. wiss. Mik.*, xiv, 1897, p. 92) is practised thus: Portions of tissue, dissected out and kept in contact with the air, are dusted (on both sides if possible) with methylen blue in fine powder. The dye dissolves in the liquids of the tissues and penetrates into them. After half an hour or so you wash in salt solution and bring into a fixing liquid. The retina, or slices of brain, may be treated in this way; see *Central Nervous System.*

351. Fixation of the Stain.—The stain obtained by any of these methods is so unstable that, as above explained, it begins to discharge after a short time, even in the living and not yet totally impregnated tissue. It may, however, be fixed, and more or less permanent preparations be made by one or other of the following methods:

Dogiel (*Arch. mik. Anat.*, xxxiii, 4, 1889, pp. 440, *et seq.*), following Arnstein (*Anat. Anzeig.*, 1887, p. 551), brings the preparations, in order to fix the colour, into saturated aqueous solution of picrate of ammonia, in which they are allowed to remain for half an hour or more, and are then removed, washed in fresh picrate of ammonia solution, and studied in dilute glycerine, or mounted permanently in glycerine saturated with picrate of ammonia. More recently (*Zeit. wiss. Mik.*, viii, 1891, p. 15) he has recommended an increased duration of the picrate of ammonia bath up to eighteen or twenty-four hours, and mounting, without washing out, in chemically pure glycerin, free from acid (this, however, has been found to extract the colour). There is a defect in this process, namely, that picrate of ammonia has a very injurious action, of a macerating nature, on some tissues. This may be avoided by adding to the fixing-bath 1 to 2 per cent. of a 1 per cent. osmic acid solution. (If it be desired to harden the tissues so that sections may be cut, the proportion of osmium solution should be increased four-fold.)

S. Mayer (*ibid.* vi, 1889, p. 422) preferred a mixture of equal parts of glycerin and saturated picrate of ammonia solution, which served to fix the colour and mount the preparations in. This was also in principle the method followed by RETZIUS (*Intern. Monatsschr. Anat. u. Phys.*, vii, 1890, p. 328). DOGIEL, after careful study, refuses to admit that this is an improvement.

Other workers have employed saturated solution of iodine in iodide of potassium (so ARNSTEIN) or picro-carmine (so FEIST, *Arch. Anat. Entwickel.*, 1890, p. 116; cf. *Zeit. wiss. Mik.*, vii, 1890, p. 231), the latter having the advantage of preserving the true blue of the stain if it be not allowed to act too long, and the preparation be mounted in pure glycerin.

Picric acid has been used by LAVDOWSKY, but this too, after careful study, is rejected by DOGIEL.

APÁTHY (*op. cit.*, § 350) brings preparations either into a concentrated aqueous solution of picrate of ammonia *free from picric acid*, and containing five drops of concentrated *ammonia* for every 100 c.c.; or, which is generally preferable, into a 1 to 2 per cent. freshly prepared solution of neutral *carbonate of ammonia* saturated with picrate. They remain in either of these solutions, preferably in the dark, for *at least an hour*. They are then brought into a *small* quantity of saturated solution of picrate of ammonia in 50 per cent. glycerin, where they remain until thoroughly saturated. They are then removed into a saturated solution of the picrate in a mixture of 2 parts 50 per cent. glycerin, 1 part cold saturated sugar solution, and 1 part similarly prepared gum-arabic solution. When thoroughly penetrated with this they are removed and mounted in the following gum-syrup medium (*loc. cit.*, p. 37):

Picked gum-arabic	50 grms.
Cane-sugar (not candied) . .	50 ,,
Distilled water	50 ,,

Dissolve over a water-bath and add 0·05 grm. thymol. This mounting medium sets quickly and as hard as balsam, so that no cementing of the mounts is necessary. Farrants' medium (with omission of the arsenious acid) will also do. In neither case should either ammonium picrate or methylen blue be added to the medium. Preparations that have been *fully* differentiated (§ 350) do not keep more than a few weeks; whilst those in which the differentiation has not been carried to the point of thorough tinctorial isolation of the "neuro-fibrils" have kept for five or six years (Apáthy, *Mitth. Zool. Stat. Neapel*, xii, 1897, p. 712).

Pleschko (*Anat. Anz.*, xiii, 1897, p. 16) fixes with picrate, and then puts into 10 per cent formol for a few days.

The methods described next § are also available for material not destined to be sectioned.

352. Methods for Sections.—The preceding methods do not give preparations that will resist the operations necessary for imbedding in paraffin or mounting in balsam. A strong solution of platinum chloride is said to give a fixation that will resist the treatment necessary for imbedding either in celloidin or paraffin (see Feist, *Arch. Anat. Entw.*, 1890, p. 116); but the precipitate it gives is a flocculent one, and the preparations are not very satisfactory.

For the earlier method of Parker (*Zool. Anzeig.*, 1892, p. 375) with methylal see early editions. Later *Mitth. Zool. Stat. Neapel*, xii, 1895, p. 4) he fixes the stain by dehydrating the objects in successive alcohols of 30, 50, 70, 95, and 100 per cent. strength, each containing 8 per cent. of corrosive sublimate, then brings them into a mixture of the last with an equal volume of xylol, and lastly into pure xylol.

The method of Bethe (*Arch. mik. Anat.*, xliv, 1894, p. 585), in its first form, is as follows: A solution is made of—

Molybdate of ammonium . .	1 grm.
Distilled water	10 grms.
Peroxide of hydrogen	1 grm.

On adding the peroxide a yellow colour is produced. A drop of hydrochloric acid is added (white precipitate of molybdic acid which dissolves on agitation). After staining and rinsing in salt solution the preparations are put into the molybdic solution. The solution ought not to be more than eight days old, and it is well to use it cooled to zero. The preparations are left in it for two or three hours if they are small, or four or five hours if they are large (of a centimetre in size). They are then washed for from half an hour to two hours in water, dehydrated in alcohol (which it is well to use cooled to zero), and cleared in clove oil, or, better, in xylol. They must then be imbedded either in paraffin or celloidin in the usual way.

This is for tissues of Vertebrates. For Invertebrates Bethe takes one grm. of molybdate, 10 c.c. of water, and 0·5 c.c. of peroxide.

PEABODY (*Zool. Bull.*, i, 1897, p. 163; *Zeit. wiss. Mik.*, xvi, 1899, p. 73) adds a drop of 1 per cent. osmic acid.

BETHE's later method (*Anat. Anz.*, xii, 1896, p. 438) is as follows: After staining, pieces of tissue of 2 to 3 mm. thickness are treated for ten to fifteen minutes with a concentrated aqueous solution of picrate of ammonia and are then brought into a solution of 1 grm. of molybdate of ammonium, either in 20 of water, or in 10 of water and 10 of 0·5 per cent. osmic acid or 2 per cent. chromic acid; or into a solution of phosphomolybdate of sodium in the same proportions, each of these solutions having added to it 1 drop of hydrochloric acid, and if desired 1 grm. of peroxide of hydrogen. They remain in one of these solutions for three quarters to one hour (or from four hours to twelve in the osmic acid one), and are then passed through water, alcohol, xylol, balsam, or paraffin. (The objects that have been treated with one of the solutions of the sodium salt are not thoroughly resistant to alcohol, so that for them it is well to cool the alcohol to under 15° C.) Sections may be after-stained with alum carmine, or "neutral" tar colours.

Slight modifications of this method are given by DOGIEL (*Arch. mik. Anat.*, xlix, 1897, p. 772; liii, 1898, p. 237; *Zeit. wiss. Zool.*, lxvi, 1899, p. 361; and *Encycl. mik. Technik*, 1903, p. 825). He omits the peroxide, the hydrochloric acid, and the cooling. Bethe (*Zeit. wiss. Mik.*, xvii, 1900, p. 21) does not approve of these modifications.

Further modifications of the molybdenum method have been published by LEONTOWITSCH (*Intern. Monatsschr. Anat.*, xviii, 1901, p. 142).

HARRIS (*Philadelphia Medical Journ.*, May 14th, 1898) after staining rinses with water, and brings the specimens into a saturated solution of either ferrocyanide or ferricyanide of potassium which has been cooled to within a few degrees of zero (a trace of osmic acid may be added to prevent maceration). They remain therein for three to twenty-four hours, and are then washed in distilled water for an hour, and are dehydrated in absolute alcohol kept at a low temperature, cleared in xylol or cedar oil, and imbedded in paraffin.

353. Impregnation of Epithelia, Lymph-spaces, etc. (DOGIEL, *Arch. mik. Anat.*, xxxiii, 1889, p. 440, *et seq.*).—Suitable pieces of tissue (thin membranes by preference) are brought fresh into a 4 per cent. solution of methylen blue in physiological salt solution (in the *Encycl. mik. Technik*, 1903, p. 827, Dogiel gives the strength of the methylen blue as ½ to 1 per cent.). After a few minutes therein they are brought into saturated solution of picrate of ammonia, soaked therein for half an hour or more, then washed in fresh picrate of ammonia solution, and examined in dilute glycerin.

If it be wished only to demonstrate the outlines of endothelium cells, the bath in the stain should be a short one, not longer than ten minutes in general; whilst if it be desired to obtain an impregnation of ground-substance of tissue, so as to have a negative image of juice canals or other spaces, the staining should be prolonged to fifteen or thirty minutes, and it is advisable to remove the endothelial covering of the objects operated on before putting them into the stain.

If it be desired to preserve the preparations permanently, they had better be mounted in glycerin saturated with picrate of ammonia. See also § 351.

The effect is practically identical (except as regards the colour) with that of a *negative impregnation with silver nitrate.* S. MAYER (*Zeit. wiss. mik.*, vi, 1889, p. 422) stains tissues for about ten minutes in a 1 : 300 or 400 solution of methylen blue in 0·5 per cent. salt solution, rinses in salt solution, and puts up in the glycerin-picrate of ammonia mixture given § 351. The images are either positive or negative, as with silver. If the stain be brought about by injection of the colour into the vascular system, the positive impregnation is the more frequent; whilst if it have been brought about by the immersion of the tissues (cornea), a negative image is more frequently obtained.

354. Toluidin Blue or Thionin as succedanea of methylen blue.—HARRIS (*Philadelphia Med. Journ.*, May 14th, 1898) has found that there is no reaction of methylen blue that cannot be equally well obtained with toluidin blue or thionin. For staining pieces of tissue he takes :

Toluidin blue, 0·1 per cent. sol. in physiological salt solution	2 parts
Ammonium chloride, 0·25 per cent. in water	1 part
Egg albumen	1 „

For injections he uses 1 part of the dye to 1000 of physiological salt solution.

Any of the methylen blue fixing methods may be employed, and the whole technique is the same.

CHAPTER XVII.

METALLIC STAINS (IMPREGNATION METHODS).

355. The Characters of Impregnation Stains.—By impregnation is understood a mode of coloration in which a colouring matter is deposited in certain elements of tissues in the form of a more or less finely granular or sometimes even flocculent *precipitate*—the impregnated elements becoming in consequence opaque. By staining, on the other hand, is understood a mode of coloration in which the colouring matter is retained by the tissues as if in a state of *solution,* showing no visible solid particles under the microscope, the stained elements remaining in consequence transparent. But it is not necessary to draw a hard and fast line between the two kinds of coloration. Some of the metallic salts treated of in this chapter give, besides an impregnation, in some cases a true stain. And some of the dyes that have been treated of in preceding chapters give, besides a stain, a true impregnation. Methylen blue, for instance, will give in one and the same preparation an impregnation and a stain; and critical examination of most fairly successful gold chloride preparations will show that the coloration is in places of the nature of a finely divided solid deposit, in others a perfectly transparent stain.

356. Negative and Positive Impregnations.—Impregnations are distinguished as *negative* and *positive.* In a negative impregnation intercellular substances alone are coloured, the cells themselves remaining colourless or very lightly tinted. In a positive impregnation the cells are stained and the intercellular spaces are unstained. (This explanation is the more needful as a directly contrary statement is made in a recent *Lehrbuch.*)

Negative impregnation is generally held to be *primary* because it is brought about by the direct reduction of a metal in the intercellular spaces. Positive impregnation is held to be *secondary* (in the case of silver nitrate at least) because it is brought about by the solution in the liquids of the tissues of the metallic deposit formed by a primary or negative impregnation, and the consequent staining of the cells by the new solution of metallic salt thus formed. These secondary impregnations take place when the reduction of the metal in the primary impregnation is not sufficiently energetic (see on these points HIS, *Schweizer Zeit. Heilk.*, ii, Heft 1, p. 1; GIERKE, *Zeit. wiss. Mik.*, i, p. 393; RANVIER, *Traité*, p. 107).

357. Action of Light on Solutions of Metallic Salts.—Stock solutions of metallic salts are generally kept in the dark, or at least in coloured bottles, under the belief that exposure to light spoils them by precipitating the metal in a state of reduction. It has been pointed out in § 33 that in the case of osmic acid, not light, but dust is the reducing agent, and that solutions may be exposed to light with impunity if dust be absolutely denied access to them. I have now good evidence to the effect that the same is the case with other metallic solutions; and the interesting point is raised whether such solutions are not positively improved for impregnation purposes by exposure to light! Dr. LINDSAY JOHNSON has been examining this question both from a histological and from a photographic point of view, and writes me as follows:

"One may (I find by experiment) state as a rule without exception that all the solutions of the chlorides and nitrates of the metals will keep indefinitely in clean white stoppered bottles in the sunlight; and as far as osmium, uranium, gold and silver, and platinum are concerned, actually improve or ripen by a good sunning. All photographers tell me their papers salt more evenly by old well-sunned silver nitrate than by a fresh solution kept in the dark; and I go so far as to say that this is one of the reasons why gold stains are so unsatisfactory."

APÁTHY (*Mitth. Zool. Stat. Neapel*, xii, 1897, p. 722) leaves his gold solutions exposed to light, so long as there are no tissues in them.

350. State of the Tissues to be impregnated.—The majority of histological stains given by dyes are only obtained with tissues that have been changed in their composition by the action of fixing and preservative reagents. With metallic impregnations the case is different; perfectly *fresh* tissues—that is such as are either living, or at all events have not been treated by any reagent whatever—will also impregnate with the greatest ease and precision. Indeed, some sorts of impregnations will not succeed at all with tissues that are not fresh in the sense above explained.

Silver.

359. Silver Nitrate: Generalities.—This is the most commonly used salt of silver. The general principles of its employment are given by RANVIER (*Traité*, p. 105) as follows:

Silver nitrate may be employed either in solution or in the solid state. The latter method is the less frequently employed, but is easy and gives good results. It is useful for the study of the cornea and of fibrous tissue, but is not suitable for epithelia. For the cornea, for instance, proceed as follows: The eye having been removed, a piece of silver nitrate is quickly rubbed over the anterior surface of the cornea, which is then detached and placed in distilled water; it is then brushed with a camel's hair brush in order to remove the epithelium. The cornea is then exposed to the action of light. On subsequent examination it will be found that the silver nitrate, which was dissolved by the liquid that bathes the surface of the cornea, has traversed the epithelium and soaked into the fibrous tissue, on the surface of which it is reduced by the action of light. The cells of the tissues will be found unstained.

Silver nitrate is generally employed in solution in the following manner: A 1 per cent. solution is taken, to which two, three, or four volumes of water are added. In the case of a membrane such as the epiploön, the membrane must be stretched like a drum-head over a porcelain dish,* and washed with distilled water, in order to remove the albuminates and white blood corpuscles that are found on its surface; it is then washed with the solution of silver nitrate. In order to obtain a powerful stain it is necessary that this part of the operation be performed in direct sunlight, or at least in a very brilliant light. As soon as the tissue has become white, and has begun to turn of a blackish grey, the membrane is removed, washed in distilled water, and mounted on a slide in some suitable examination medium.

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* **The Hoggans Histological Rings** will be found much more convenient. They are vulcanite rings made in pairs, in which one ring just fits into the other, so as to clip and stretch pieces of membrane between them. They will be found described and figured in *Journ. Roy. Mic. Soc.*, ii, 1879, p. 357, and in ROBIN's *Journ. de l'Anat.*, 1879, p. 54. They may be obtained, in sets of various sizes, of Burge & Warren, 42, Kirby Street, Hatton Garden, London, E.C.

If the membrane were left in the water the cells would become detached, and would not be found in the finished preparation.

If the membrane had not been stretched as directed the silver would be precipitated not only in the intercellular spaces, but in all the small folds of the surface, and the forms of the cells would be disguised.

If the membrane had not been washed with distilled water before impregnation there would have been formed a deposit of silver on every spot on which a portion of an albuminate was present, and these deposits might easily be mistaken for a normal structure of the tissue. It is thus that impurities in the specimen have been described as stomata of the tissue.

If the solution be taken too weak—for instance, 1 : 500 or 1 : 1000, or if the light be not brilliant—a *general* instead of an *interstitial* stain will result; nuclei will be most stained, then protoplasm, and the intercellular substance will contain but very little silver. In general in a good "impregnation" the contents of the cells, and especially nuclei, are quite invisible.

The tissues should be constantly *agitated* in the silver-bath in order to avoid the formation on their surfaces of deposits of chlorides and albuminates of silver, which would give rise to deceptive appearances.

These impregnations only succeed with *fresh* tissues, and cannot be made to succeed with tissues preserved in any way.

360. Silver Nitrate: the Solutions to be employed (Ranvier).—The solutions generally employed by Ranvier vary in strength from 1 : 300 to 1 : 500. Thus 1 : 300 is used for the epiploön, pulmonary endothelium, cartilage, tendon; whilst a strength of 1 : 500 is employed for the study of the phrenic centre, and for that of the epithelium of the intestine. For the impregnation of the endothelium of blood-vessels (by injection) solutions of 1 : 500 to 1 : 800 are taken.

M. DUVAL (*Précis*, p. 229) recommends solutions of 1, 2, or at most 3 per cent.

v. RECKLINGHAUSEN used, for the cornea, a strength of from 1 : 400 to 1 : 500 (*Die Lymphgefässe*, etc., Berlin, 1862, p. 5).

ROBINSKI (*Arch. de Physiol.*, 1869, p. 451) used solutions varying between 0·1 and 0·2 per cent., which he allowed to act for thirty seconds.

REICH (*Sitzb. wien. Akad.*, 1873, Abth. 3, April) takes solutions of from 1 : 600 to 1 : 400 for the study of the endothelium of vessels by injection.

ROUGET (*Arch. de Physiol.*, 1873, p. 603) employed solutions as weak as 1 : 750, or even 1 : 1000, exposing the tissues to their action several times over, and washing them with water after each bath.

The HERTWIGS take, for marine animals, a 1 per cent. solution (*Jen. Zeit. Naturk.*, xvi, pp. 313 and 324).

The HOGGANS (*Journ. of Anat. and Physiol.*, xv, 1881, p. 477) take for lymphatics a 1 per. cent. solution.

TOURNEUX and HERRMANN (ROBIN'S *Journal de l'Anat.*, 1876, p. 200) for the epithelia of Invertebrates employed a solution of 3 : 1000 strength, and in some cases weaker solutions. The tissues were allowed to remain in the silver-bath for one hour, and were washed out with alcohol of 90 per cent.

HOYER (*Arch mik. Anat.*, 1876, p. 649) takes a solution of nitrate of silver of known strength, and adds ammonia to it until the precipitate that is formed just redissolves, then dilutes the solution until it contains from 0·75 to 0·50 per cent. of the salt.

This *ammonio-nitrate* solution is intended principally for the impregnation of the endothelium of vessels by injection, but can also be used for the impregnation of membranes by pouring on. It has the advantage of impregnating absolutely nothing but endothelium or epithelium; connective tissue is not affected by it.

RANVIER'S injection-mass for impregnating endothelium is given § 504.

DEKHUYSEN (*Anat. Anz.*, iv, 1889, No. 25, p. 789) has applied to tissues of terrestrial animals the method of HARMER for marine animals (see § 365). For details see *previous editions.*

REGAUD (*Journ. Anat. et Phys.*, xxx, 1894, p. 719) recommends for the study of lymphatics a process devised by RENAUT, for the details of which see also *previous editions.*

361. Other Salts of Silver.—ALFEROW (DUVAL, *Précis*, p. 230) recommends the picrate, lactate, acetate, and citrate, as giving better results than the nitrate. He employs them in solutions of 1 : 800, and adds to the solution employed for staining a small quantity of the acid of the salt taken (10 to 15 drops of a concentrated solution of the acid to 800 c.c. of the solution of the salt). The object of the free acid is to decompose the precipitates formed by the action of the silver salt on the chlorides, carbonates, and other substances existing in the tissues.

362. Silver Nitrate: Reduction.—Reduction may be effected in other media than distilled water.

v. RECKLINGHAUSEN washed his preparations in salt solution before exposing them to the light in distilled water (*Arch. path. Anat.*, xix, p. 451). Physiological salt solution (0·75 per cent.) is commonly used for these washings.

MÜLLER (*Arch. f. path. Anat.*, xxxi, p. 110), after impregnation by immersion for two or three minutes in a 1 per cent. solution of nitrate of silver in the dark, adds to the solution a small quantity of 1 per cent. solution of iodide of silver (dissolved by the aid of a little iodide of potassium). After being agitated in this mixture the preparations are washed with distilled water, and exposed to the light for two days in a 1 per cent. solution of nitrate of silver (see also GIERKE, in *Zeit. wiss. Mik.*, i, 1884, p. 396).

ROUGET (*Arch. de Physiol.*, 1873, p. 603) reduces in glycerin.

SATTLER (*Arch. Mik. Anat.*, xxi, p. 672) exposes to the light for a few minutes in water acidulated with acetic or formic acid. THANHOFFER (*Das Mikroskop*, 1880) employs a 2 per cent. solution of acetic acid.

KRAUSS brings his preparations, after washing, into a light red solution of permanganate of potash. Reduction takes place very quickly, even in the dark. The method does not always succeed (see GIERKE, in *Zeit. wiss. Mik.*, i, 1884, p. 400).

OPPITZ brings his preparations for two or three minutes into a 0·25 or 0·50 per cent. solution of chloride of tin. Reduction takes place very rapidly (GIERKE, *loc. cit.*).

JAKIMOVITCH (*Journ. de l'Anat.*, xxiii, 1888, p. 142) brings nerve preparations, as soon as they have become of a dark brown colour, into a mixture of formic acid 1 part, amyl alcohol 1 part, and water 100 parts. The objects exposed to the light in this mixture for two or three days at first become brighter, a part of the reduced silver being dissolved: hence the mixture must be renewed from time to time. When all the silver has dissolved, a darker colour is permanently assumed. The nerve-cells are left in this mixture for five to seven days.

DEKHUYSEN (*op. cit.*, last §) reduces in oil of cloves, after dehydration.

363. Fixation.—LEGROS (*Journ. de l'Anat.*, 1868, p. 275) washes his preparations, after reduction, in hyposulphite of soda, which he says prevents after-blackening. According to DUVAL (*Précis*, p. 230) they should be washed for a few seconds only in 2 per cent. solution, and then in distilled water.

GEROTA (*Arch. Anat. Phys., Phys. Abth.*, 1897, p. 428) greatly recommends reduction in a hydroquinone developing solution, followed by fixation in hyposulphite of soda, just as in photography.

364. Nature of the Metallic Deposit.—As to the nature of the black or brown deposit or stain formed in the intercellular spaces in cases of primary impregnation see SCHWALBE, *Arch. mik. Anat.*, vi, 1870, p. 5; GIERKE'S *Färberei zu mikroskopischen Zwecken*, in vols. i and ii of *Zeit. wiss. Mik.*; JOSEPH, *Sitzb. Akad. Wiss. Berlin*, 1888; *Zeit. wiss. Mik.*, xi, 1, 1894, p. 42 *et seq.* It evidently cannot consist of metallic silver, as it is soluble in hyposulphite of soda; but it may be an albumino-nitrate, or an oxide of silver.

365. Silver Impregnation of Marine Animals.—On account of the chlorides that bathe the tissues of marine animals, these cannot be treated *directly* with nitrate of silver.

HERTWIG (*Jen. Zeit.*, xiv, 1880, p. 322) recommends fixing them with a weak solution of osmic acid, then washing with distilled water until the wash-water gives no more than an insignificant precipitate with silver nitrate, and then treating for six minutes with 1 per cent. solution of silver nitrate.

HARMER (*Mitth. Zool. Stat. Neapel*, v, 1884, p. 445) washes them for some time (half an hour) in a 5 per cent. solution of nitrate of potash in distilled water; they may then be treated with silver nitrate in the usual way.

Harmer thinks that for some animals other solutions having the same density as sea water might be substituted for the nitrate of potash, and recommends a 4·5 per cent. solution of sulphate of soda.

366. Impregnation of Nerve Tissue.—For this subject, which includes the important bichromate-and-silver method of Golgi, see Part II.

367. Double-staining Silver-stained Tissues.—The nuclei of tissues impregnated with silver may be stained with the usual reagents, provided that solutions containing free ammonia be avoided, as this would dissolve out the silver. These stains will only succeed, however, with successful negative impregnations, as nuclei that have been impregnated will not take the second stain.

Impregnation with silver may be followed by impregnation with gold. In this case the gold generally substitutes itself for the silver in the tissues, and though the results are sharp and precise, the effect of a double stain is not produced. See hereon Gerota, *loc. cit.*, § 363.

Gold.

368. The Characters of Gold Impregnations.—Gold chloride differs from nitrate of silver in that it generally gives *positive* (§ 356) impregnations only. It generally gives negative images only when caused to act on tissues that have first received a negative impregnation with silver, the gold substituting itself for the silver. In order to obtain these images you first impregnate very lightly with silver; reduce; treat for a few minutes with a 0·5 per cent. solution of gold chloride, and reduce in acidulated distilled water.

This process, however, is in but little use, and except for certain special studies on the cornea, and on connective tissue, the almost exclusive function of gold chloride is the impregnation of nervous tissue. For this tissue, gold chloride exhibits a remarkable selectivity, in virtue of which it justly ranks as a most valuable reagent for the study of nerve end-organs and the distribution of nerves.

For all the objects above named gold chloride is capable of furnishing preparations that for beauty and clearness cannot be surpassed, if even they can be equalled by any other means. But it is very uncertain in its action.

It is now acknowledged that the very best gold preparations give images that are only worthy of credence as to what they show, and furnish absolutely no evidence whatever as to the non-existence of anything that they do not show; for you can never be sure that the imbibition of the salt has not failed, or its reduction stopped at any point. That the images frequently *do* stop capriciously short in the representation of reality there is abundant evidence.

369. Pre-impregnation and Post-impregnation. — Gold methods may be divided into two groups; the one, chiefly concerned with the study of *peripheral* nerves or nerve end-organs, is characterised by employing either *perfectly fresh* tissues or tissues that have been subjected to a special treatment by organic acids; the other, concerned chiefly with the study of nerve-*centres*, is characterised by the employment of *fixed and hardened* tissues.

These two groups of methods may be distinguished with APÁTHY as the *pre-impregnation* methods (*Vorvergoldung*), and the *post-impregnation* methods (*Nachvergoldung*). They give in some respects opposite results. Pre-impregnation gives nuclei unstained, cytoplasm rather strongly stained, axis-cylinders reddish-violet. Post-impregnation gives nuclei sharply stained, cytoplasm pale, axis-cylinders black, and (when successful) showing their neurofibrils sharply distinguished from the interfibrillar substance.

In APÁTHY'S view (*Mitth. Zool. Stat. Neapel*, xii, 1897, p. 718) successful gold preparations should show a true *stain*, not an impregnation (§ 355), the stain being brought about by the formation of gold oxide (AuO) which combines with the tissue elements. He advises in consequence that preparations should *not be moved about* more than can be helped in the reducing bath, so that the colouring oxide may not be washed away from the tissues before the stain has taken effect.

370. As to the Commercial Salts of Gold.—SQUIRE's *Methods and Formulæ,* etc. (p. 43) says: "Commercial chloride of gold is not the pure chloride, $AuCl_3$, but the crystallised double chloride of gold and sodium, containing 50 per cent. of metallic gold.

"Commercial chloride of gold and sodium is the above crystallised double chloride mixed with an equal weight of chloride of sodium, and contains 25 per cent. of metallic gold."

This, however, appears not to be the case in Germany. Dr. GRÜBLER, writing to MAYER (see the *Grundzüge,* LEE und MAYER, p. 215) says: "*Aurum chloratum fuscum* contains about 53 per cent. Au, the *flavum* about 48 per cent.; in both of them there should be only water and hydrochloric acid besides the gold, no sodium chloride. Pure *Auronatrium chloratum* contains 14·7 per cent. of sodium chloride, though samples are found in commerce with much more."

APÁTHY (*Mitth. Zool. Stat. Neapel,* xii, 1897, p. 722) formerly employed the *aurum chloratum flavum,* but now prefers the *fuscum.*

A. *Pre-impregnation.*

371. The State of the Tissues to be impregnated.—The once classical rule, that for researches on nerve-endings the tissues should be taken perfectly fresh, seems not to be valid for all cases. For DRASCH (*Sitzb. Akad. Wiss. Wien,* 1881, p. 171, and 1884, p. 516; and *Abhand. math.-phys. Cl. K. Sach. Ges. Wiss.,* xiv, No. 5, 1887; *Zeit. wiss. Mik.,* iv, 1887, p. 492) finds that better results are obtained with tissues that have been allowed to lie after death for twelve, twenty-four, or even forty-eight hours in a cool place. He even suspects that the function of the organic acids in the methods inspired by LÖWIT's method is to bring the tissues into somewhat the state in which they are naturally found at a certain moment of post-mortem process—a state, namely, in which the nerves have a special susceptibility for impregnation with gold.

372. COHNHEIM's **Method** (*Virchow's Arch.,* Bd. xxxviii, pp. 346—349; *Stricker's Handb.,* p. 1100). Fresh pieces of cornea (or other tissue) are put into solution of chloride of gold of 0·5 per cent. strength until they are thoroughly yellow, and then exposed to the light in water acidulated with acetic acid until the gold is thoroughly reduced, which happens in the course of a few days at latest. They are then mounted in acidulated glycerin.

The method in this, its primitive form, often gave splendid results, but was very uncertain, giving sometimes a nuclear or protoplasmic stain, sometimes an extra-cellular impregnation similar to that of nitrate of silver. And the preparations thus obtained are anything but permanent.

373. Löwit's **Method** (*Sitzgsber. Akad. Wien,* Bd. lxxi, 1875, p. 1).—The principle of this process is that, in order to facilitate the penetration of the gold and its subsequent reduction in the tissues, the tissues are made to swell up by treatment with formic acid before being brought into the gold-bath, and formic acid is employed to assist the reduction after impregnation.

The following directions, which may serve as a type, are taken from Fischer's paper on the corpuscles of Meissner (*Arch. mik. Anat.,* xii, 1875, p. 366).

Small pieces of *fresh* skin are put into dilute formic acid (one volume of water to one of the acid of 1·12 sp. gr.), and remain there until the epidermis peels off. They then are put for fifteen minutes into gold chloride solution (1½ per cent. to 1 per cent.), then for twenty-four hours into dilute formic acid (1 part of the acid to 1—3 of water), and then for twenty-four hours into undiluted formic acid. (Both of these stages are gone through in the dark). Thin sections are then made and mounted in dammar or glycerin. Successful preparations show the nerves alone stained.

374. Ranvier's **Formic Acid Method** (*Quart. Journ. Mic. Sci.* [N. S.], lxxx, 1880, p. 456).—Reflecting that the action of the one third formic acid in which Löwit placed his tissues must be hurtful to the final ramifications of the nerves, Ranvier combines the formic acid with a fixing agent designed to antagonise its altering action, and takes for this purpose the chloride of gold itself. The tissues are placed in a mixture of chloride of gold and formic acid (four parts of 1 per cent.

gold chloride to one part of formic acid) which has been boiled and allowed to cool (Ranvier's *Traité*, p. 826). They remain in this until thoroughly impregnated (muscle twenty minutes, epidermis two to four hours) ; the reduction of the gold is effected either by the action of daylight in acidulated water, or in the dark in dilute formic acid (one part of the acid to four parts of water).

The object of boiling the mixture of gold chloride and formic acid is this, that " by boiling in the presence of the acid the gold acquires a great tendency to reduction, and for this reason its selective action on nervous tissues is enhanced."

375. Ranvier's **Lemon-juice Method** (*Traité*, p. 813).—Ranvier finds that of all acids lemon juice is the least hurtful to nerve-endings. He therefore soaks pieces of tissue in fresh lemon juice, filtered through flannel, until they become transparent (five or ten minutes in the case of muscle). They are then rapidly washed in water, brought for about twenty minutes into 1 per cent. gold chloride solution, washed again in water, and brought into a bottle containing 50 c.c. of distilled water and two drops of acetic acid. They are exposed to the light for twenty-four to forty-eight hours. The preparations thus obtained are good for immediate study, but are not permanent, the reduction of the gold being incomplete. In order to obtain perfectly reduced, and therefore permanent, preparations, the reduction should be done in the dark in a few cubic centimetres of dilute formic acid (1 part acid to 4 of water). The reduction is complete in twenty-four hours.

376. Viallane's **Osmic Acid Method** (*Hist. et Dév. des Insectes*, 1883, p. 42).—The tissues are treated with osmic acid (1 per cent. solution) until they begin to turn brown, then with 25 per cent. formic acid for ten minutes ; they are then put into solution of chloride of gold of 1 : 5000 (or even much weaker) for twenty-four hours in the dark, then reduced in the light in 25 per cent. formic acid. According to my experience this is an excellent method.

377. Other Methods.—The numerous other methods that have been proposed differ from the foregoing partly in respect of the solutions used for impregnation, but chiefly in respect of details imagined for the purpose of *facilitating the reduction* of the gold, and rendering it as complete as possible.

Thus BASTIAN modified COHNHEIM'S original method by employing a solution of gold chloride of a strength of 1 to 2000, acidulated with HCl (1 drop to 75 c.c.), and performing the reduction in a mixture of equal parts of formic acid and water *kept warm,* heat being an agent that furthers reduction.

HÉNOCQUE (*Arch. de l'Anat. et de la Physiol.,* 1870, p 111) impregnates in a 0·5 per cent. solution of gold chloride, washes in water for twelve to twenty-four hours, and reduces, with the aid of heat, in a nearly saturated solution of *tartaric acid.* The best temperature for reduction is 40° to 50° C. Reduction is effected very rapidly, sometimes in a quarter of an hour.

This process has been described as the method of CHRSCHTSCHONOWIC (*Arch. mik. Anat.,* vii, 1872, p. 383).

HOYER (*ibid.,* ix, 1873, p. 222) says that the double chloride of *gold and potassium* has the following advantages over the simple gold chloride. It is more easy to be obtained of unvarying composition, it is more perfectly neutral, and its solutions are more perfectly stable. He impregnates in solutions of 0·5 per cent. strength, and reduces in water containing one or two drops of a *pyrogallic acid* developing solution, such as is used in photography, or in a warm concentrated solution of tartaric acid, kept at the temperature of an incubating stove until the gold is fully reduced.

I have myself used the double chloride of *gold and sodium* with good results.

CIACCIO (*Journ. de Microgr.,* vii, 1883, p. 38) prefers the double chloride of *gold and cadmium.*

FLECHSIG (*Die Leitungsbahnen in Gehirn,* 1876; *Arch. Anat. u. Phys.,* 1884, p. 453) reduces in a 10 per cent. solution of *caustic soda.*

NESTEROFFSKY treats impregnated preparations with a drop of *sulphydrate of ammonium,* and finishes the reduction in glycerin (quoted from GIERKE'S *Färberei z. mik Zwecken*).

BÖHM reduces in *Pritchard's solution*—amyl alcohol, 1; formic acid, 1; water, 98.

Manfredi treats fresh tissues as follows (*Arch. per le Sci. med.*, v, No. 15) : Gold chloride, 1 per cent. half an hour ; *oxalic acid*, 0·5 per cent., in which they are warmed in a water-bath to 36°, allowed to cool, and examined. Mount in glycerin. Sunny weather is necessary.

Boccardi (*Lavori Instit. Fisiol. Napoli*, 1886, i, p. 27 ; *Journ. Roy. Mic. Soc.*, 1888, p. 155) recommends oxalic acid of 0·1 per cent. or of 0·25 to 0·3 per cent., or a mixture of 5 c.c. pure formic acid, 1 c.c. of 1 per cent. oxalic acid, and 25 c.c. of water. Objects should remain in this fluid in the dark not longer than two to four hours.

Kolossow (*Zeit. wiss. Mik.*, v, 1888, p. 52) impregnates for two or three hours in a 1 per cent. solution of gold chloride acidulated with 1 per cent. of HCl, and reduces for two or three days in the dark in a 0·01 per cent. to 0·02 per cent. solution of *chromic acid*.

Geberg (*Intern. Monatsschr.*, x, 1893, p. 205) states that previous treatment of tissues for twenty-four hours with *lime-water* (Arnstein's method) greatly helps the reduction, see § 668.

Bernheim (*Arch. Anat. Phys., Phys. Abth.*, 1892, Supp., p. 29) adds to Löwit's dilute formic acid a piece of *sulphite of sodium* (must be fresh and smell strongly of sulphurous acid).

Dr. Lindsay Johnson writes to me that besides the "sunning" of the impregnating solution recommended above (§ 357), the following precautions should be taken : "The tissue must be well washed in distilled water, and the gold carefully *acidulated with a neutral acetate* or formiate, or acetic or formic acid, at least twenty-four hours before using ; and then afterwards the tissue must be washed until no reaction occurs to test-paper."

Apáthy (*Mikrotechnik*, p. 173 ; *Mitth. Zool. Stat. Neapel*, xii, 1897, pp. 718—728) lays stress on the necessity of having the objects *thoroughly penetrated by light* from all sides during the process of reduction. Objects, therefore, should always be so thin that light can readily stream through them ; they should either be membranes or sections. They should be either stretched out (*e.g.* on a slide) or hung up in the reducing bath in such a way as to be lighted from both sides. He impregnates for a few hours in 1 per cent. gold chloride (§ 370) in the dark, then brings the objects without washing out with water, the gold solution being just superficially mopped up with blotting-paper, into 1 per cent. formic acid. They are to be set up in this, in a tube or otherwise, so that the light *may come through them from all sides,* and exposed to diffused daylight in summer, or direct sunlight in winter, for six to eight hours *without a break.* They must *not be moved about* more than can be helped in the acid. If the acid becomes brown it may be changed for fresh. The temperature of the acid should not be allowed to rise over 20° C., whence direct sunlight is to be avoided during the summer. He mounts in glycerin or his syrup (§ 351). He finds such preparations absolutely permanent.

Post-impregnation.

378. GERLACH'S **Method** (STRICKER'S *Handb.,* 1872, p. 678): Spinal cord is hardened for fifteen to twenty days in a 1 to 2 per cent. solution of bichromate of ammonia. Thin sections are made and thrown into a solution of 1 part of double chloride of gold and potassium to 10,000 parts water, which is very slightly acidulated with HCl. They remain there from ten to twelve hours, and having become slightly violet, are washed in hydrochloric acid of 1 to 2 : 3000 strength, then brought for ten minutes into a mixture of 1 part HCl to 1000 parts of 60 per cent. alcohol, then for a few minutes into absolute alcohol, and thence into clove oil, for mounting in balsam.

(See further, for Nerve Centres, under "Neurological Methods.")

379. GOLGI (*Mem. Accad. Torino* [2], xxxii, 1880, p. 382) treats tissues previously hardened in 2 per cent. solution of bichromate of potash, as follows: They are put for ten to twenty minutes into 1 per cent. solution of arsenic acid, then into ½ per cent. solution of chloride of gold and potassium for half an hour, washed in water, and reduced in sunlight in 1 per cent. arsenic acid solution, which is changed for fresh as fast as it becomes brown. Mount in glycerin. Sunny weather is necessary.

380. APÁTHY'S **Method** (*Zeit. wiss. Mik.,* x, 1893, p. 349;

Mitth. Zool. Stat. Neapel, xii, 1897, p. 729). The material to be used must have been fixed either in sublimate or in a mixture of equal parts of saturated solution of sublimate in 0·5 per cent. salt solution and 1 per cent. osmic acid (this more particularly for Vertebrates). The material should be imbedded *as quickly as possible*, either in paraffin or in celloidin. The paraffin material will keep in a good state indefinitely, and so will the celloidin material provided that the blocks be preserved in a thick solution of glycerin jelly with a piece of thymol in it (the jelly is removed before cutting by warming and washing with warm water). Sections are made when desired and fixed on slides, and after the usual treatment with iodine, etc., are either put into distilled water for from two to six hours, or are rinsed in water, treated for one minute with 1 per cent. formic acid, and again well washed with water.

They are then put for twenty-four hours, or at least overnight, into the gold-bath, which is preferably 1 per cent. gold chloride (see § 370), but may be weaker, down to 0·1 per cent., after which they are just rinsed with water or superficially dried with blotting-paper. The slides are then set up on end in a sloping position, the sections looking downwards, so that precipitates may not fall on them, in glass tubes filled with 1 per cent. formic acid. The tubes are then exposed to light until the gold is reduced, as directed in § 377, *sub. fin.* (you may set them up near a window, and place a reflector of some sort behind them; a sheet of white paper will do). After trial I highly recommend this process. I have found it advantageous to reduce in weak solution of *formaldehyde*, either with or without formic acid. A few drops of formol added to the tube with the objects will suffice.

381. Impregnation of Marine Animals.—For some reason the tissues of marine animals do not readily impregnate with gold in the fresh state. It is said by Fol that impregnation succeeds better with spirit specimens.

382. Preservation of Impregnated Preparations.—Preparations may be mounted either in balsam or in acidulated glycerin (1 per cent. formic acid).

Theoretically they ought to be permanent if the reduction of the metal has been completely effected, but they are very liable to go wrong through after-blackening. RANVIER states that this can be avoided by putting them for a few days into alcohol, which he says possesses the property of stopping the reduction of the gold.

Blackened preparations may be *bleached* with cyanide or ferricyanide of potassium. REDDING employs a weak solution of ferricyanide, CYBULSKY a 0·5 per cent. solution of cyanide. But the results are far from being perfectly satisfactory.

Preparations may be double-stained with the usual stains (safranin being very much to be recommended), but nuclei will only take the second stain in the case of negative impregnation.

Other Metallic Stains.

383. Osmic Acid and Pyrogallol.—This method was first published by me in 1887 (*La Cellule,* iv, p. 110). It consists in putting tissues that have been treated with osmic acid into a weak solution of pyrogallol, in which they quickly turn greenish black, sometimes much too much so.

HERMANN (*Arch. mik. Anat.,* xxxvii, 4, 1891, p. 570) obtained the reaction with tissues fixed in his platino-aceto-osmic mixture (§ 50). He put tissues for one or two days into the platino-aceto-osmic mixture, washed thoroughly in water, and hardened in successive alcohols; after which, to obtain the black reaction, he put for twelve to eighteen hours into raw *pyroligneous* acid. This acid ought (*Ergebnisse der Anat.,* ii, 1893, p. 28) to be as raw as possible, and to be of a dark brown colour and evil-smelling. (The stain obtained in this way is *not* due to a mere reduction of the osmic acid, but also to coloration by the brown pyroligneous acid; for HERMANN has obtained the same stain with sublimate material, or alcohol material [*op. cit.,* i, 1891 (1892), p. 7]).

According to my experience, the procedure of HERMANN gives much better results than the pure osmic acid process, but not the best possible. I now proceed as follows:

Either the mixture of HERMANN or the mixture of FLEMMING may be used for fixing. In the interest of the stain alone, half an hour therein is enough and *is preferable* to a longer immersion. It is not only useless but hurtful to put the preparations into alcohol after fixation, for it is desirable that the tissue should be in as *fresh* a state as possible on coming into the pyrogallol. In consequence it is not possible to obtain the best results by treating paraffin sections. The tissues are therefore brought in bulk, directly after fixing, into a weak aqueous solution of *pyrogallol*. The tissues may remain in it for twenty-four hours, but for small objects an hour or less is sufficient. An *alcoholic* solution of pyrogallol *may* be taken if desired.

There is thus obtained a black stain, which is at the same time a plasma stain and a nuclear stain, chromatin being so far stained that it is not necessary to have recourse afterwards to a special chromatin stain. With Invertebrates it sometimes gives very elegant differentiations of nervous tissue. It is a *very easy* method, and if pyrogallol be used a very *safe* one (with pyroligneous acid not so safe).

If it be desired to add a chromatin stain, I greatly recommend safranin (stain very strongly, twenty-four hours at least, and start the extraction with acid alcohol).

This method has been attributed to VON MAEHRENTHAL. See also a modification of this method by AZOULAY, under "Neurological Methods." See also a similar process by HELLER and GUMPERTZ, quoted *Zeit. wiss. Mik.*, xii, 1896, p. 385; and one by KOLOSSOW (*ibid.*, ix, 1892, p. 38, and ix, 1893, p. 316).

384. Perchloride of Iron.—This reagent, introduced by POLAILLON (*Journ. de l'Anat.*, iii, 1866, p. 43), sometimes gives useful results, especially in the study of peripheral nerve-ganglia, in which it stains the nervous tissue alone, the connective tissue remaining colourless.

The HOGGANS proceed as follows (*Journ. Quekett Club*, 1876; *Journ. Roy. Mic. Soc.*, ii, 1879, p. 358):—The tissue (having been first fixed with silver nitrate, which is somewhat reduced by a short exposure to diffused light) is dehydrated in alcohol, and treated for a few minutes with 2 per cent. solution of perchloride of iron in spirit; then with a 2 per cent. solution of pyrogallic acid in spirit, and in a few minutes more, according to the depth of tint required, may be washed in water and mounted in glycerin.

FOL fixes in perchloride (§ 85) and treats for twenty-four hours with alcohol containing a trace of gallic acid.

POLAILLON (*loc. cit.*) reduces in tannic acid.

This method is not applicable to chromic objects.

Roosevelt (*Med. Rec.*, ii, 1887, p. 84; *Journ. Roy. Mic. Soc.*, 1888, p. 157) employs a stain composed of 20 drops of saturated solution of iron sulphate, 30 grms. water, and 15 to 20 drops pyrogallic acid.

385. Palladium Chloride (see Schulze, § 82). **Prussian Blue** (see Leber, *Arch. Ophthalm.*, xiv, p. 300; Ranvier, *Traité*, p. 108). **Cupric Sulphate** (see Leber, *ibid.*). **Lead Chromate** (see Leber, *ibid.*). **Sulphides** (see Landois, *Centralb. med. Wiss.*, 1885, No. 55; and Gierke, in *Zeit. wiss. Mik.*, i, 1884, p. 497). **Molybdate of Ammonia** (Merkel; Krause) (see Gierke, *ibid.*, i, 1884, p. 96). **Oxychloride of Ruthenium** (Nicolle and Cantacuzène) (see *Ann. Inst. Pasteur*, vii, 1893, p. 331). **Ruthenium Red** (Ruthenium Sesquichloride) (Eisen, *Zeit. wiss. Mik.*, xiv, 1897, p. 200; in my hands totally useless).

CHAPTER XVIII.

OTHER STAINS AND COMBINATIONS.

386. Kernschwarz (PLATNER, *Zeit. wiss. Mik.*, iv, 1887, p. 350).—A black liquid imported from Russia by Grübler and Hollborn. Its exact composition is unknown, but MAYER (*Grundzüge*, p. 202) finds that it contains a metallic base, namely iron, combined with an organic acid, which is highly probably some gallic acid. I use it as follows:

Sections (I have not tried material in bulk) are fixed on slides and treated with Kernschwarz until the required depth of stain is obtained, which will be from a few minutes to twenty-four hours, according to the material.

There is obtained a black or neutral-tint stain, which is, according to the previous treatment of the material, either a pure chromatin stain, or at the same time a plasma stain. If overstaining should have occurred, the stain is easily differentiated by means of any weak acid, either in water or alcohol. PLATNER took alkalies, preferably carbonate of lithia, for differentiation; but that is clearly faulty practice.

It may be well, if a good plasma stain has been obtained, to after-stain for twenty-four hours with safranin, followed by differentiation in either neutral or acid alcohol, and clove oil. The stain is perfectly permanent in balsam, and is stated to be a good one for preparations that it is desired to photograph.

I greatly recommend this stain, which is safe and easy.

387. Brazilin, the colouring matter of Brazilian redwood or Pernambuco wood, has been recommended by EISEN (*Zeit. wiss. Mik.*, xiv, 1897, p. 198), and HICKSON (*Nature*, lxii, 1900, p. 589, and *Quart. Journ. Mic. Sci.*, 1901, p. 469). MAYER (*Grundzüge*, p. 203) finds that it gives a stain similar to that of hæmatein, but much weaker, and is therefore at the least superfluous.

388. Orchella (Orseille) see WEDL (*Arch. path. Anat.*, lxxiv, p. 143); and FOL (*Lehrb.*, p. 192), and early editions of this work.

389. Orcein (ISRAEL, *Virchow's Archiv*, cv, 1886, p. 169; and *Prakticum der path. Hist.*, 2 Aufl., Berlin, 1893, p. 72).—Orcein is a dye obtained from the lichen, *Lecanora parella*, and is not to be confused with *orcin*, another derivative of the same lichen. It is said to unite in itself the staining properties of the basic and acid stains, and also the combination of two contrast colours. Israel stains sections in a solution containing 2 grms. of orcein, 2 grms. of glacial acetic acid, and 100 c.c. of distilled water, washes in distilled water, and passes rapidly through absolute alcohol to thick cedar oil, in which the preparations remain definitely mounted. Nuclei blue, protoplasm red.

See also "Connective Tissues" in Part II.

390. Purpurin, see RANVIER'S *Traité technique*, p. 280; DUVAL'S *Précis de Technique histologique*, p. 221; and GRENACHER'S formula in *Arch. mik. Anat.*, xvi, 1879, p. 470. A very weak stain.

391. Indigo.—Indigo is employed in histology in the form of solutions of so-called indigo-carmine, or sulphindigotate of soda or potash. The simple aqueous solution gives a diffuse stain, and is therefore not capable of being usefully employed *alone*. It is, however, of use when employed to bring about a *double* stain in conjunction with carmine, see below.

Thiersch's Oxalic Acid Indigo-carmine (see *Arch. mik. Anat.*, i, 1865, p. 150).

392. Other Superfluous Vegetal Dyes.—See *early editions*. To this category belong those recommended by CLAUDIUS (*Zeit. wiss. Mik.*, xvii, 1900, p. 52).

Carmine Combinations.

393. Seiler's Carmine followed by Indigo-Carmine (*Am. Quart. Mic. Journ.*, i, 1879, p. 220; *Journ. Roy. Mic. Soc.*, ii, 1879, p. 613).—Stain in borax-carmine, wash out with HCl alcohol, wash out the acid, and after-stain in an *extremely dilute* alcoholic solution of indigo-carmine (two drops of saturated aqueous solution added to an ounce of alcohol and filtered).

I find this method gives good results when applied to sections, but very bad results if it be attempted to stain in bulk.

394. Merkel's Carmine and Indigo-Carmine in One Stain (MERKEL, *Unters. anat. Anst. Rostock*, 1874; *Month. Mic. Journ.*, 1877, pp. 242 and 317).

Also NORRIS and SHAKESPEARE, *Amer. Journ. Med. Sci.*, January, 1877; MERKEL, *Mon. Mic. Journ.*, 1877, p. 242; MARSH, *Section Cutting*, p. 85; BAYERL, *Arch. mik. Anat.*, xxiii, 1885, pp. 36, 37; MACALLUM, *Trans. Canad. Instit.*, ii, 1892, p. 222; *Journ. Roy. Mic. Soc.*, v, 1892, p. 698.

MERKEL's formula, as has been pointed out by MAYER (*Mitth. Zool. Stat. Neapel*, xii, 2, 1896, p. 320), is not only highly irrational and inconvenient to employ, but gives an alkaline fluid that may be injurious to tissues.

395. P. MAYER's **Carmalum (or Hæmalum) and Indigo-Carmine in One Stain.**—MAYER (*loc. cit.*, last §) obtains very good results by taking a solution of 0·1 gramme of indigo-carmine in 50 c.c. of distilled water, or 5 per cent. alum solution, and combining it with from four to twenty volumes of carmalum or hæmalum.

396. Carmine and Picro-Indigo-Carmine (RAMÓN Y CAJAL, *Rev. de Cienc. med.*, 1895; CALLEJA, *Rev. trim. Microgr.*, ii, 1897, p. 101; *Zeit. wiss. Mik.*, xv, 1899, p. 323).—For use after a carmine stain, CAJAL takes a solution of 0·25 gramme of indigo-carmine in 100 grammes saturated aqueous solution of picric acid. Stain (*sections*) for five to ten minutes, wash in weak acetic acid, then in water, then remove the excess of picric acid with absolute alcohol, clear and mount. Employed in this way, the indigo is said to give a sharper plasma stain than without the picric acid.

RAMÓN Y CAJAL also (*Elementos de Histologia*, 1897; quoted from *La Cellule*, xix, 1901, p. 212) employs the picro-indigo mixture after **Magenta**; stain strongly in saturated solution of magenta, rinse in water until no more colour comes away, and pass into the indigo mixture. Said to be "the best of all methods for epithelia and connective tissue." See also BORREL, *Ann. Inst. Pasteur*, 1901, p. 57, or LEE et HENNEGUY, *Traité*, p. 268.

397. Carmine and Anilin Blue (or Bleu Lumière, or Bleu de Lyon) (DUVAL, *Précis de Technique Microscopique*, 1878, p. 225).—Stain with carmine; dehydrate, and stain for a few minutes (ten minutes for a section of nerve-centres) in an alcoholic solution of anilin blue (ten drops of saturated solution of anilin blue soluble in alcohol to ten grammes of absolute alcohol, for sections of nerve-centres). Clear with turpentine, without further treatment with alcohol, and mount in balsam.

Other authors recommend, instead of anilin blue, bleu de

Lyon, dissolved in 70 per cent. alcohol acidulated with acetic acid (MAURICE and SCHULGIN), or bleu lumière.

The solutions of both these colours should be extremely dilute for sublimate material, but strong for chrom-osmium material. It is possible to use them for staining in bulk.

BAUMGARTEN (*Arch. mik. Anat.*, xl, 1892, p. 512) stains sections (of material previously stained in borax-carmine) for twelve hours in a 0·2 per cent. solution of bleu de Lyon in absolute alcohol, and washes out for about half that time before mounting in balsam. He recommends the process for cartilage and nerve-centres.

398. Carmine and Malachite Green.—MAAS (*Zeit. wiss. Zool.*, l, 4, 1890, p. 527) recommends borax-carmine followed by weak alcoholic solution of malachite green, with a final washing out with stronger alcohol. See also § 325.

399. Carmine and Picro-nigrosin (PIANESE). See *Journ. Roy. Mic. Soc.*, 1892, p. 292.

400. Carmine and Picric Acid. See § 308.

Hæmatein or Hæmatoxylin Combinations.

401. Hæmatoxylin and Picric Acid.—See § 308.

402. Hæmatoxylin and Eosin or Benzopurpurin (§ 316).—Objects may be stained with hæmatoxylin (either in the mass or as sections) and the sections stained for a few minutes in eosin. I think it is better to take the eosin weak, though it has been recommended (STÖHR, see *Zeit. wiss. Mik.*, i, 1884, p. 583) to take it saturated. Either aqueous or alcoholic solutions of eosin may be used.

HICKSON (*Quart. Journ. Mic. Sci.*, 1893, p. 129) stains sections for one hour in a strong solution of eosin in 90 per cent. alcohol, washes with alcohol, and stains for twenty minutes in a weak solution of hæmatoxylin.

This method is most particularly recommendable for embryological sections, as vitellus takes the eosin stain energetically, and so stands out boldly from the other germinal layers in which the blue of the hæmatoxylin dominates.

See also LIST (*Zeit. wiss. Mik.*, ii, 1885, p. 148); BUSCH

(*Verh. Berl. Phys. Ges.*, 1887); Gierke (*Zeit. wiss. Mik.*, i, 1884, p. 505).

Sections should be very well washed before being passed from eosin into hæmatoxylin or the reverse, as eosin very easily precipitates hæmatoxylin.

403. Renaut's **Hæmatoxylic Eosin** (Fol's *Lehrbuch*, p. 196). A very complicated glycerin mixture, which acts so slowly that it may take weeks to stain, and, I think, superfluous.

Everard, Demoor, and Massart (*Ann. Inst. Pasteur*, vii, 1893, p. 166) prepare a similar mixture as follows: A solution is made with 1 grm. of eosin, 25 grms. of alcohol, 75 of water, and 50 of glycerin. Then 20 grms. of alum are dissolved by the aid of heat in 200 grms. of water, the solution is filtered, and after twenty-four hours there is added to it 1 grm. of hæmatoxylin dissolved in 10 grms. of alcohol. This solution is allowed to stand for eight days, then filtered again, and combined with an equal volume of the eosin solution.

404. Hæmatoxylin and Congo.—See § 314.

405. Hæmatoxylin and Safranin.—Rabl (*Morph. Jahrb.*, x, 1884, p. 215) stained *very lightly* with *very dilute* Delafield's hæmatoxylin for twenty-four hours, then for some hours in (Pfitzner's) safranin, and washed out with pure alcohol. The plasma stain is here given by the hæmatoxylin.

406. Hæmatoxylin and Säurefuchsin.—Stain first with iron hæmatoxylin or hæmalum, then stain (sections) in 0·5 per cent. aqueous solution of Säurefuchsin, dehydrate and mount.

407. Hæmatoxylin and Säurefuchsin and Orange.—Proceed as above, using for the second stain the following mixture: Säurefuchsin, 1 grm.; orange, 6 grms.; rectified spirit, 60 c.c.; water, 240 c.c. (from Squire's *Methods and Formulæ*, p. 42). Using orange G (not mentioned by Squire), I have had very good results.

The process described by Cavazzani (*Riforma Med.*, Napoli, 1893, p. 604; *Zeit. wiss. Mik.*, xi, 3, 1894, p. 344) is far too complicated to be recommendable.

408. Hæmatoxylin and Picro-Säurefuchsin (van Gieson, *New York Med. Journ.*, 1889, p. 57; quoted from Moeller, *Zeit. wiss. Mik.*, xv, 2, 1898, p. 172, which see for further details).

Proceed as above, using for the second stain the picro-Säurefuchsin mixture, § 309. The second stain must not be too prolonged or the hæmatoxylin stain may be attacked.

Weigert (*Zeit. wiss. Mik.*, xxi, 1904, p. 1) stains first in his iron-hæmatoxylin mixture (§ 263), rinses in water, and stains for a short time in his picro-Säurefuchsin (§ 309), rinses, dehydrates with 90 per cent. alcohol, and clears with carbolic acid-xylol mixture (§ 179).

CHAPTER XIX.

EXAMINATION AND PRESERVATION MEDIA.

409. Introductory.—I comprehend under this heading all the media in which an object may be examined. The old distinction of "indifferent" liquids, and those which have some action on tissues, appears to be misleading more than helpful; for *no* medium is without action on tissues except the plasma with which they are surrounded during the life of the organism; and this plasma itself is only "indifferent" whilst all is *in situ*; as soon as a portion of tissue is dissected out and transferred to a slide in a portion of plasma the conditions become artificial.

It does not appear necessary to create a separate group for mounting media, as all preservative media may be used for mounting, though the only media that will afford an *absolutely sure* preservation of soft tissues are the resinous ones.

For directions as to making permanent mounts in fluid media see the early sections of Chap. XX.

Watery Media.

410. Water.—Water may be employed for the examination of structures that have been *well fixed*; but it is by no means applicable to the examination of fresh tissues. It is very far from being an "indifferent" liquid; many tissue elements are greatly changed by it (nerve-end structures, for instance), and some are totally destroyed by its action if prolonged (for instance, red blood corpuscles).

411. Isotonic and "Indifferent" Liquids.—In order to render water inoffensive to fresh tissues it must have dissolved in it substances of similar diffusibility to those of the liquids of the tissue, so as to prevent the occurrence of osmosis, to which process the destructive action of pure water is mainly due. Now cell contents are a mixture of colloids and crystalloids; consequently, in order to reduce osmotic processes to a minimum, it is necessary that the examination medium contain a due proportion of both crystalloids and colloids. By adding, for instance, white of egg to salt solution this end may be in some measure attained; and, as a matter of fact, the liquids recommended as "indifferent" are generally found to contain both crystalloids and colloids. Liquids thus composed, in which tissue-elements are in osmotic equilibrium—that is, neither swell nor shrink—are said to be *isotonic* to the tissues; whilst those in which they shrink are called *hypertonic,* and those in which they swell *hypotonic.* Solutions of common salt, in different concentrations, form the base of the most commonly employed isotonic liquids. For marine Invertebrates, sea-water is generally isotonic.

412. Salt Solution ("normal salt solution," "physiological salt solution").—0·75 per cent. sodium chloride in water. CARNOY recommends the addition of a trace of osmic acid.

According to LOCKE (*Boston Med. Surg. Journ.*, 1896, p. 514) there should be added to salt solution (which to be isotonic should contain, according to HAMBURGER, 0·9 to 1 per cent. of salt)—0·01 per cent. chloride of potassium, and 0·02 per cent. chloride of calcium, in order to obtain an "indifferent" liquid.

MALASSEZ (*C. R. Soc. Biol.*, iii, 1896, pp. 504 and 511) takes for erythrocytes about 1 per cent. sodium chloride.

DEKHUYSEN (*Onderz. Phys. Lab. Leiden.*, 1900, p. 149) takes for blood of *Rana* 0·8 per cent.

For *Selachians,* MUSKENS (*Tijd. Nederb. Dierk. Ver.*, 1894, p. 314) finds 2¼ per cent. right; and RODIN (*Comptes. Rend.*, 1900, p. 1009) 1·5 to 2·6 per cent., according to the species.

ENGELMANN (*Deutsch. med. Wochenschr.*, xxix, 1903, p. 64), finds that 0·9 per cent. is isotonic with human blood-serum, and 0·64 per cent. for red blood corpuscles of the frog.

413. PICTET'S **Liquid** (*Mitth. Zool. Stat. Neapel*, x, 1891, p. 89).—5 to 10 per cent. solution of chloride of manganese. These proportions are for *marine* animals, and for terrestrial animals will generally be found much too high. For these from 1 per cent. to 3 per cent. will be nearer the mark. I find this liquid excellent.

414. Aqueous Humour, Simple White of Egg.—Require no preparation beyond filtering. They may be iodised if desired (see next §), or mixed with salt solution.

415. Iodised Serum.—First recommended by Max Schultze (*Virchow's Archiv*, xxx, 1864, p. 263). I take the following from Ranvier (*Traité*, p. 76).

The only serum that gives really good results is the amniotic liquid of mammals. A gravid uterus of a sheep or cow having been obtained (in large slaughterhouses such can be obtained without difficulty), an incision is made through the wall of the uterus and the fœtal membranes. A jet of serum issues from the incision, and is caught in a flask prepared for the purpose. Flakes of iodine are then added, and the flask is frequently agitated for some days. A perfectly fresh amnios must be taken, for the merest incipience of putrefaction will spoil the preparation. The flask should have a wide bottom, so that the serum may form only a shallow layer in it; otherwise the upper layers will not be sufficiently exposed to the action of the iodine.

Another method is as follows: Serum is mixed with a large proportion of tincture of iodine; the precipitate of iodine that forms is removed by filtration, and there remains a strong solution of iodine in serum. This should be kept in stock, and a little of it added every two or three days to the serum that is intended for use.

Ranvier explains that at the outset serum dissolves very little iodine; but if an excess of iodine be kept constantly present in the solution, it will be found that after two or three weeks iodides are formed, and allow fresh quantities of iodine to dissolve; so that after one or two months a very strongly iodised serum is obtained. It should be dark brown. Such a solution is the most fitting for the purpose of iodising fresh serum in the manner directed above, and for making the different strengths of iodised serum that are required for different purposes. In general for maceration purposes a serum of a pale brown colour should be employed.

416. Artificial Iodised Serum (Frey, *Das Mikroskop*, 6 Aufl., 1877, p. 75).

Distilled water	270 grms.
White of egg	30 „
Sodium chloride . . .	2·5 „

Mix, filter, and add tincture of iodine.

417. KRONECKER'S **Artificial Serum** (from VOGT et YUNG, *Traité d'Anat. Comp. Prat.*, p. 473).

Common salt	6 grms.
Caustic soda	0·06 grm.
Distilled water	1000 grms.

BÖHM und OPPEL (*Taschenbuch*, 3 Aufl., p. 19) take carbonate of soda instead of caustic soda.

418. MIGULA'S **Glycerized Blood-serum** (see the paper in *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 172).

419. **Chloride of Calcium** (HARTING, *Das Mikroskop*, 2 Aufl., p. 297).—The aqueous solution, either saturated or diluted with 4 to 8 parts of water, has a low refractive index and does not dry up.

420. **Acetate of Potash** (MAX SCHULTZE, *Arch. mik. Anat.*, vii, 1872, p. 180).—A nearly saturated solution in water. The index of refraction is lower than that of glycerin.

This medium has been frequently recommended as having the property of preventing the blackening of objects that have been treated with osmium; but it seems extremely doubtful whether this is really the case.

421. **Syrup.**—A good strength for syrup is equal parts of loaf sugar and water. Dissolve by boiling. To preserve it from mould, chloral hydrate may be dissolved in it (1 to 5 per cent.)—I have used as much as 7 per cent., and found no disadvantage—or carbolic acid may be employed instead of chloral; 1 per cent. is sufficient.

It may be used as a mounting medium, but is not to be recommended for that purpose, as there is always risk of the sugar crystallising out.

FABRE-DOMERGUE (*Bull. Soc. Philomath*, ix, 1899, p. 115) dissolves 200 parts of sugar in 400 of water, and adds 1 part of formaldehyde, and camphor to saturation.

422. **Chloral Hydrate.**—5 per cent. in water (LADOWSKY, *Arch. f. mik. Anat.*, 1876, p. 359).

Or, 2·5 per cent. in water (BRADY, *British Copepods*).

Or, 1 per cent. in water (MUNSON, *Journ. Roy. Mic. Soc.*, 1881, p. 847).

Mercurial Liquids.

(*I give these as examination media only, not as permanent mounting media. Media containing sublimate always end by making tissues granular.*)

423. GILSON'S **Fluid** (CARNOY'S *Biologie cellulaire*, p. 94).

Alcohol of 60 per cent.	60 c.c.
Water	30 „
Glycerin	30 „
Acetic acid (15 parts of the glacial to 85 of water)	2 „
Bichloride of mercury	0·15 grm.

424. GAGE'S **Albumen Fluid** (*Zeit. f. wiss. Mik.*, 1886, p. 223).

White of egg	15 c.c.
Water	200 „
Corrosive sublimate	0·5 grm.
Salt	4 grms.

Mix, agitate, filter, and preserve in a cool place. Recommended for the study of red blood corpuscles and ciliated cells.

425. PACINI'S **Fluids** (*Journ. de Mic.*, iv, 1880; *Journ. Roy. Mic. Soc.*, [N. S.], ii, 1882, p. 702, and early editions of this work).—Antiquated and superfluous. They consist essentially of corrosive sublimate of from one half to one third per cent. strength, with the addition of a little salt or acetic acid.

426. GOADBY'S **Fluids** (*Micro. Dict.*, art. "Preservation," or early editions of this work).—Quite unsuited for histological purposes.

Other Fluids.

427. Chloride and Acetate of Copper (RIPART et PETIT'S fluid, *Brebissonia*, 1880, p. 92; CARNOY'S *Biol. Cell.*, p. 95).

Camphor-water (not saturated)	75 grms.
Distilled water	75 „
Crystallised acetic acid	1 grm.
Acetate of copper	0·30 „
Chloride of copper	0·30 „

A *valuable* medium for work with delicate fresh tissues.

It may be used in combination with methyl green, *which it does not precipitate.* The addition of a drop of osmic acid or corrosive sublimate does not cause the least turbidity, and enhances its *fixing* action.

428. Tannin (*loc. cit.*).

Water	100 grms.
Powdered tannin	0·40 grm.

As an examination medium only.

429. WICKERSHEIMER'S **Fluid** (*Zool. Anz.*, 1879, p. 670).—Worthless for histological purposes.

430. Medium of FARRANTS (BEALE, *How to Work*, etc., p. 58).

Picked gum arabic	4 ounces.
Water	4 „
Glycerin	2 „

To be kept in a stoppered bottle with a lump of camphor. Slightly different receipts for this are given by the *Micrographic Dictionary*, and A. F. STANLEY KENT, in *Journ. Roy. Mic. Soc.*, 1890, p. 820.

431. Gum and Glycerin Medium (LANGERHANS, *Zool. Anzeig.*, ii, 1879, p. 575).

Gummi arab.	5·0
Aquæ	5·0

To which after twelve hours are added—

Glycerini	5·0
Sol. aquosa acid. carbol. (5·100)	10·0

432. ALLEN'S **Gum and Glycerin.**—Prof. F. J. Allen (*in. litt.*) recommends solution of gum arabic of the consistency of glycerin to be strained, and one eighth volume of glycerin and one twentieth of formol gradually incorporated. Sets hard.

433. HOYER'S **Gum with Chloral Hydrate or Acetate of Potash** (*Biol. Centralb.*, ii, 1882, pp. 23, 24).—A high 60 c.c. glass with a wide neck is filled two thirds full with gum arabic (in pieces), and then *either* a solution of chloral (of several per cent.) containing 5—10 per cent. of glycerin is added *or* officinal solution of acetate of potash or ammonia. Filter after solution. The solution with chloral is for carmine or hæmatoxylin objects—that with acetate for anilin objects.

434. COLE'S **Gum and Syrup Medium.** See § 196.

435. APÁTHY'S **Gum and Syrup Medium** (see § 351).—This medium is recommended by APÁTHY for mounting in general.

It sets very hard and, combined with a paper cell (see § 465), may be used for ringing glycerin mounts.

436. Fabre-Domergue's **Glucose Medium** (*La Nature*, No. 823, 9 Mars, 1889, supp.):

Glucose syrup diluted to twenty-five degrees of the areometer (sp. gr. 1·1968)	1000 parts.
Methyl alcohol	200 ,,
Glycerin	100 ,,
Camphor to saturation.	

The glucose is to be dissolved in warm water, and the other ingredients added. The mixture, which is always acid, must be neutralised by the addition of a little potash or soda.

This medium is said to preserve without change almost all *animal pigments.*

437. Brun's **Glucose Medium** (from Fabre-Domergue's *Premiers Principes du Microscope et de la Technique microscopique*, Paris, 1889, p. 123).

Distilled water	140 parts.
Camphorated spirit	10 ,,
Glucose	40 ,,
Glycerin	10 ,,

Mix the water, glucose, and glycerin, then add the spirit, and filter to remove the excess of camphor which is precipitated on mixing. Dr. Henneguy informs me that this liquid preserves the colour of preparations stained with anilin dyes, *methyl green included.*

438. Levulose is recommended as a mounting medium by Behrens, Kossel u. Schiefferdecker (*Das Mikroskop, etc.*, 1889). It is uncrystallisable, and preserves well carmine and coal-tar stains (hæmatoxylin stains fade somewhat in it). The index of refraction is somewhat higher than that of glycerin. Objects may be brought into it out of water.

Glycerin Media.

439. Glycerin.—Glycerin diluted with water is frequently employed as an examination and mounting medium. Dilution with water is sometimes advisable on account of the increased visibility that it gives to many structures. But from the point of view of efficacious preservation it is always advisable to use undiluted glycerin, the strongest that can be procured (see BEALE, *How to Work*, etc.).

For closing glycerin mounts, the edges of the cover should first (after having been cleansed as far as possible from superfluous glycerin) be painted with a layer of *glycerin jelly*; as soon as this is set a coat of any of the usual cements may be applied. See next chapter.

Glycerin dissolves carbonate of lime, and is therefore to be rejected in the preparation of calcareous structures that it is wished to preserve.

440. Extra-refractive Glycerin.—The already high index of refraction of glycerin (Price's glycerin, $n = 1{\cdot}46$) may be raised to about that of crown glass by dissolving suitable substances in the glycerin. Thus the refractive index of a solution of chloride of cadmium ($CdCl_2$)* in glycerin may be 1·504; that of a saturated solution of sulphocarbolate of zinc† in glycerin may be 1·501; that of a saturated solution of SCHERING's‡ chloral hydrate (in crusts) in glycerin is 1·510; that of iodate of zinc in glycerin may be brought up to 1·56.§ The clearing action of glycerin may thus be greatly increased, and homogenous objectives used to greater advantage. For further details see *previous editions*.

441. Glycerin and Alcohol Mixtures.—These afford one of the best means of bringing delicate objects gradually from weak into strong glycerin. The object is mounted in a drop of the liquid, and left for a few hours or days, the mount not being closed. By the evaporation of the alcohol the liquid gradually increases in density, and after some time the mount may be closed, or the object brought into pure glycerin or glycerin jelly.

1. CALBERLA'S LIQUID (*Zeit. wiss. Zool.*, xxx, 1878, p. 442).

Glycerin	1 part.
Alcohol	2 parts.
Water	3 ,,

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* *Journ. Roy. Mic. Soc.*, ii, 1879, p. 346.

† *Ibid.*, iii, 1880, p. 1051.

‡ *Ibid.*, (N. S.), i, 1881, p. 943.

§ *Ibid.*, p. 366.

2. I strongly recommend the following for very delicate objects :

Glycerin	1 part.
Alcohol	1 „
Water	2 parts.

3. Hantsch's Liquid.

Glycerin	1 part.
Alcohol	3 parts.
Water	2 „

4. Jäger's Liquid (Vogt and Yung's *Traité d'Anat. comp. parat.*, p. 16).

Glycerin	1 part.
Alcohol	1 „
Sea water	10 parts.

Glycerin Jellies.

442. **Glycerin Jellies** have a higher index than pure glycerin, and set hard enough to make luting unnecessary, though it is well to varnish the mount. To use them, you melt a small portion on a slide, introduce the object (previously soaked in water or glycerin), and cover. They seem very plausible, but for delicate work I do not recommend them, and should advise instead either pure glycerin or balsam.

443. Lawrence's **Glycerin Jelly** (Davies, *Preparation and Mounting of Microscopic Objects*, p. 84).—"He takes a quantity of Nelson's gelatin, soaks it for two or three hours in cold water, pours off the superfluous water, and heats the soaked gelatin until melted. To each fluid ounce of the gelatin, *whilst it is fluid but cool*, he adds a fluid drachm of the white of an egg. He then boils this until the albumen coagulates and the gelatin is quite clear, when it is to be filtered through fine flannel, and to each ounce of the clarified solution add 6 drachms of a mixture composed of 1 part of glycerin to 2 parts of camphor water."

444. Brandt's **Glycerin Jelly** (*Zeit. wiss. Mik.*, ii, 1880, p. 69).—Melted gelatin 1 part, glycerin 1½ parts.

The gelatin to be soaked in water and melted in the usual way. After incorporating the glycerin, the mixture is to be filtered. Swedish filtering paper does not allow the fluid to pass through sufficiently, and flannel produces more threads than before. BRANDT filters through spun glass pressed into the lower part of a funnel. He describes a simple arrangement for keeping the funnel warm during the filtering (see *early editions*).

Some drops of carbolic acid should be added to the fluid product of the filtering.

445. KAISER's **Glycerin Jelly** has been given § 164.

446. SQUIRE's **Glycerin Jelly** (SQUIRE's *Methods and Formulæ*, etc., p. 84).—Soak 100 grms. of French gelatin in chloroform water, drain when soft, and dissolve with heat in 750 grms. of glycerin. Add 400 grms. of chloroform water with which has been incorporated about 50 grms. of fresh egg-albumen ; mix thoroughly, and heat to boiling-point for about five minutes. Make up the total weight to 1550 grms. with chloroform water. Filter in a warm chamber.

447. GILSON's **Chloral Hydrate Jelly** (communicated by Prof. GILSON).—1 vol. of gelatin, melted *secundum artem*, and 1 vol. of Price's glycerin. Mix, and add 1 vol. of chloral hydrate (*i.e.* add crystals of chloral until the volume of the mixture has increased by one half) ; warm till dissolved. This gives a very *highly refractive* medium, which is found useful for opaque tissues that it is desired not to dehydrate.

A similar medium is published by GEOFFROY, *Journ. de Botan.*, 1893, p. 55 (see *Zeit. wiss. Mik.*, ix, 1893, p. 476). He dissolves, by the aid of as little heat as possible, 3 to 4 grms. of gelatin in 100 c.c. of 10 per cent. aqueous solution of chloral hydrate.

High Refractive Liquids.

448. STEPHENSON's **Biniodide of Mercury and Iodide of Potassium** (*Journ. Roy. Mic. Soc.* [N. S.], ii, 1882, p. 167).—A solution of the two salts in water. "This is very easily prepared by adding the two salts to the water until each shall be in excess ; when this point of saturation has been reached the liquid will be found to have a refractive index of 1·68." Any lower index can be obtained by suitable dilution with water.

This fluid is very dense, its specific gravity being 3·02. It is highly antiseptic.

"For marine animals a weak solution is probably well adapted, as about a 1 per cent. solution (5 minims to the ounce) will give the specific gravity of sea water, with no appreciable difference in the refractive index."

Covers should be sealed with white wax, and the mounts finished with two or three coatings of gold-size and one of shellac.

I have experimented both with strong and with weak solutions. They are not adapted, I find, for the purposes of a *permanent* mounting medium. Tissues are well preserved, but the preparations are ruined by a precipitate which forms in the fluid. But as a temporary examination medium I have occasionally found this solution valuable. Its optical properties are wonderful; it allows of the examination of watery tissues, *without any dehydration,* in a medium of refractive index surpassing that of any known resinous medium.

See further details in *early editions.*

449. Monobromide of Naphthalin.—See *Journ. Roy. Mic. Soc.*, 1880, p. 1043 (ABBE and VAN HEURCK), and *Zool. Anz.*, 1882, p. 555 (MAX FLESCH).

Resinous Media.

450. Resins and Balsams.—Resins and balsams consist of a vitreous or amorphous substance held in solution by an essential oil. By distillation or drying in the air they lose the essential oil and pass into the solid state. It is these solidified resins that should, in my opinion (and that, I believe, of the best microscopists) be employed for microscopical purposes; for the raw resins always contain a certain proportion of water, which makes it difficult to obtain a clear solution with the usual menstrua, is injurious to the optical properties of the medium and to its preservative qualities, and, further, especially hurtful to the preservation of stains. I therefore do not share the contrary opinion expressed by FOL (*Lehrb.*, pp. 138—9), but recommend that all solutions be made by heating gently the balsam or resin in a stove until it becomes brittle when cold, and then dissolving in an appropriate menstruum. Solid resins are now easily found in commerce.

Solutions made with volatile menstrua, such as xylol and chloroform, set rapidly, but become rapidly brittle. Solutions made with non-volatile media, such as turpentine, set much less rapidly, and pass much less rapidly into the brittle state. The latter should therefore be employed whenever it is above all desired to have a mount that will prove as durable as possible.

Another motive is that turpentine media preserve the *index of visibility* of the preparations much longer than do media made with volatile menstrua. Preparations made with these last become so transparent in course of time that much fine detail is often lost. (Such mounts may, however, be revivified without removing the cover by putting them for a day or two into a tube of benzol; the benzol penetrates the balsam, and brings it down to a lower refractive index.) The *visibility* of minute *colourless* structures is proportional to the *difference* between the refractive indices of the object and of the medium in which it is mounted. The majority of the elements of soft tissues are (after fixation) of an index of refraction somewhat superior to that of Canada balsam. It follows that by lowering the index of the balsam increased visibilty is obtained, and the desideratum in any case is to find a medium just low enough to give good *visibility*, and yet not so low as to injure the performance of homogeneous objectives.

Turpentine colophonium is a safe and excellent medium, but is injurious to alum-hæmatein stains. For these, and in general where a more strongly clearing medium is desired, *xylol balsam* is about the most recommendable, though it is not *perfectly* safe, the mounts sometimes developing granules. Seiler's *alcohol balsam* is a fine medium, and perfectly stable. *Oil of cedar* is sometimes useful, it keeps perfectly, and with time it thickens sufficiently to hold the cover in place; or if desired, preparations may be luted with Bell's cement. After using an oil immersion objective on a fresh mount, it is always easy to change the cover by floating it up with a drop of the oil placed at the side.

451. Canada Balsam.—Prepare with the solid balsam as described § 450. The usual menstrua are xylol, benzol, chloroform, and turpentine. Turpentine has the advantages pointed out § 450, but the defect that it does not always give a homogeneous solution with Canada balsam, as it does with colophonium. For most purposes the xylol solution is the best. If time be an object, a benzol solution should be preferred, as it sets much quicker than the xylol solution. The chloroform solutions become very brown with age, and are injurious to stains made with tar dyes. Benzol is good when chemically pure and *free from water.*

SAHLI (*Zeit. wiss. Mik.*, ii, 1885, p. 5) dissolves in cedar oil.

Samples of balsam that are *acid* are frequently met with, and are injurious to some stains. Grübler & Hollborn now prepare a *neutral balsam*, in which MAYER has found that very delicate preparations, that lost colour immediately in any other sort of balsam, have kept perfectly for many months. For a process of neutralising balsam with carbonate of soda or potash see COLUCCI (*Giorn. Ass. Med. Natural Napoli*, vii, 1897, p. 172).

452. SEILER'S **Alcohol Balsam** (*Proc. Amer. Soc. Mic.*, 1881, pp. 60–2; *Journ. Roy. Mic. Soc.* [N.S.], ii, 1882, pp. 126-7). —Dissolve solid balsam in warm absolute alcohol, and filter through absorbent cotton.

Objects may be mounted in it direct from absolute alcohol, without previous treatment with an essential oil or other clearing agent; SEILER considers that by this means "shrivelling is avoided, as well as *the solution of fat in the cells.*"

The process of mounting direct from alcohol is rather ticklish. But used in the ordinary way, after clearing by an essence, or by xylol or the like, SEILER'S solution is for most purposes admirable.

It is *one of the most stable solutions known to me.* Care should be taken not to breathe on it during the process of mounting, as this may cause cloudiness.

453. Damar (Gum Damar, or Dammar, or d'Ammar).—The menstrua are the same as for balsam. For directions for preparing solutions, by various authors, see *early editions.* After ample experience I am convinced that *not one of these solutions can be depended on for* *permanent preservation.* Sooner or later, sometimes after a few weeks or days, or it may be only after months or years, granules make their appearance in the mounts.

454. Colophonium.—A solution of colophonium in turpentine was first recommended by KLEINENBERG. Both KLEINENBERG and MAYER warn against the employment of absolute alcohol as a solvent; the preparations are beautiful at first, but soon become spoiled by the precipitation of crystals or of an amorphous substance. The turpentine solution *keeps perfectly limpid.*

To make the solution, I add small lumps of good *pale* colophonium to a quantity of rectified oil of turpentine kept in a stove, and when a sufficiently thick solution has been obtained, filter twice, the filtering being done in the stove. About a fortnight is required for the whole process. The solution should not be too thick, as it thickens with age.

This medium drys very slowly (so that ample time is afforded for arranging objects in it). In the winter a slide will take about a month before it will be hard enough to be safe with *oil-immersion lenses*; whereas an alcohol-balsam mount will be dry enough in a couple of days. It injures alum-hæmatein stains; but with these exceptions I find it *a most excellent medium.*

REHM (*Zeit. wiss. Mik.*, ix, 1893, p. 387) dissolves 1 part colophonium in 10 of *benzin*; and later writers also recommend a similar solution.

455. Venice Turpentine (VOSSELER, *Zeit. wiss. Mik.*, vi, 1889, p. 292, *et seq.*).—Commercial Venice turpentine is mixed in a tall cylinder glass, with an equal volume of 96 per cent. alcohol, allowed to stand in a warm place for three or four weeks, and decanted. It is stated that preparations may be mounted in this medium without previous clearing with essential oils or the like. Stains keep well, according to VOSSELER.

MAYER (*Grundzüge*, p. 236) notes hereon that not *all* stains will keep well in it on account of the alcohol and oil of turpentine in it; hæmalum stains fade rapidly in it. He considers it a very valuable medium on account of its faculty of supporting a notable proportion of water in the preparations. Celloidin sections can be mounted direct from 96 per cent.

alcohol; it does not cause turbidity in the albumen of MAYER's fixative for sections, and you may breathe on it with impunity whilst mounting. This faculty of withstanding moisture makes it especially valuable at the seaside.

This medium is also recommended by SUCHANNEK (*Zeit. wiss. Mik.*, vii, 1891, p. 463). He advises that it be prepared with equal parts of Venice turpentine and neutral absolute alcohol (obtained by treating commercial absolute alcohol with calcined cupric sulphate and quicklime). The mixture should be agitated frequently and kept in a tile stove for a day or two until clear and sufficiently inspissated.

456. **Thickened Oil of Turpentine** has been used as a mounting medium by some workers. To prepare it, pour some oil into a plate, cover it lightly so as to protect it from dust without excluding the air, and leave it until it has attained a syrupy consistency.

457. **Cedar Oil.**—See § 450, *sub. fin.*

458. **Gum Thus,** dissolved in xylol, is recommended by EISEN, *Zeit. wiss. Mik.*, xiv, 1897, p. 201.

459. **Styrax and Liquidambar.**—See *Journ. Roy. Mic. Soc.*, 1883, p. 741; *ib.*, 1884, pp. 318, 475, 655, and 827; and the places there quoted. Also *Bull. Soc. Belge de Mic.*, 1884, p. 178; and FOL, *Lehrb.*, p. 141. These are very highly refractive media, therefore seldom useful in histology.

460. **Sandarac** (LAVDOWSKY, from *Ref. Handbook Med. Sci.*, Supp. p. 438).—Gum sandarac 30 grs., absolute alcohol 50 c.c. According to MAYER (*Grundzüge*, p. 258) it keeps very badly.

461. **Photographic Negative Varnish** (for mounting large sections without cover-glasses).—See WEIGERT, *Zeit. wiss. Mik.*, iv, 1887, p. 209.

462. **Castor Oil.**—Recommended by GRENACHER (*Abhandl. naturf. Ges. Halle-a.-S.*, Bd. xvi; *Zeit. wiss. Mik.*, 1885, p. 244) with the idea that its low refractive index ($n = 1{\cdot}49$) would give a useful augmentation of visibility for the more refractive elements of the tissues. With the objects with which I have experimented I have not had good results.

CHAPTER XX.

CEMENTS AND VARNISHES.

463. Introduction.—Two, or at most three, of the media given below will certainly be found sufficient for all useful purposes. For many years I have used only one cement (Bell's). I recommend this both as a cement and varnish; gold size may be found useful for turning cells; and Miller's caoutchouc cement may be kept for occasions on which the utmost solidity is required.

Marine glue is only necessary for making glass cells.

For the operations of mounting in fluids, and of making cells and ringing, see Carpenter's *The Microscope.*

Carpenter lays great stress on the principle that the cements or varnishes used for fluid mounts should always be such as contain *no mixture of solid particles*; he has always found that those that do, although they might stand well for a few weeks or months, yet always become porous after a greater lapse of time, allowing the evaporation of the liquid and the admission of air. All fluid mounts *should have the edges of the cover carefully dried and be ringed with glycerin jelly before applying a cement; by this means all danger of running in is done away with.* See §§ 464 and 465.

See also Aubert, *The Microscope,* xi, 1891, 150, and *Journ. Roy. Mic. Soc.,* 1891, p. 692; Beck, *The Microscope,* xi, 1891, pp. 338, 368, and *Journ. Roy. Mic. Soc.,* 1892, p. 293; Behrens' *Tabellen zum Gebrauch bei mikroskopischen Arbeiten* (Bruhn, Braunschweig, 1892); Rousselet, *Journ. Quek. Mic. Club,* vii, 1898, p. 93; and, as to the comparative *tenacity* of divers cements, Behrens, *Zeit. wiss. Mik.,* ii, 1885, p. 54, and Aubert, *Amer. Mon. Mic. Journ.* 1885, p. 227; *Journ. Roy. Mic. Soc.,* 1886, p. 173).—Behrens gives the palm to amber varnish; Aubert places Miller's caoutchouc cement at the head of the list, Lovett's cement coming half-way down, and zinc white cement at the bottom, with less than one quarter the tenacity of the caoutchouc cement.

464. Gelatin Cement.—Marsh's *Section-cutting*, 2nd ed., p. 104).—Take half an ounce of Nelson's opaque gelatin, soak well in water, melt in the usual way, stir in 3 drops of creasote, and put away in a small bottle. It is used warm.

When the ring of gelatin has become quite set and dry, which will not take long, it may be painted over with a solution of bichromate of potash made by dissolving 10 grains of the salt in an ounce of water. This should be done in the daytime, as the action of daylight is necessary to enable the bichromate to render the gelatin insoluble in water. The cover may then be finished with Bell's cement.

This process is particularly adapted for glycerin mounts.

465. The Paper Cell Method.—By means of two punches I cut out rings of paper of about a millimetre in breadth, and of about a millimetre smaller in diameter than the cover-glass. *Moisten* the paper ring with mounting fluid, and centre it on the slide. Fill the cell thus formed with mounting fluid; arrange the object in it; put the cover on; fill the annular space between the paper and the margin of the cover with glycerin jelly (a turn-table may be useful for this operation); and as soon as the gelatin has set turn a ring of gold-size on it, and when that is quite dry, varnish with Bell's cement.

For greater safety, the gelatin may be treated with bichromate, according to Marsh's plan, last §.

466. Rousselet's **Method for Aqueous Mounts** (*op. cit.*, § 463).—Close the mount with a ring of a mixture of two parts of a solution of damar in benzol and one part gold-size. When dry, put on three or four thin coats of pure gold-size at intervals of twenty-four hours, and finish with a ring of Ward's brown cement.

467. Ward's **Brown Cement** is a shellac-alcohol solution, made by E. Ward, Oxford Road, Manchester. Its best solvent, Mr. Rousselet writes me, is a mixture of wood-naphtha and alcohol. He considers it the best shellac varnish he has met with, better than BELL's.

468. BELL's **Cement.**—Composition unknown. May be obtained from the opticians, or from J. Bell & Co., chemists, 338, Oxford Street, London.

This varnish flows easily from the brush, and sets quickly. The cover should be ringed, as above described, with glycerin jelly before applying the varnish. This precaution is especially necessary with glycerin. This varnish is soluble in ether or chloroform. It is not attacked by oil of cedar.

469. MILLER's **Caoutchouc Cement.**—Composition unknown. May be obtained from the opticians. A very tenacious and, which is frequently an important point, a quickly drying cement. It may be diluted by a mixture of equal parts of chloroform and strong alcohol (see ROUSSELET, *Journ. Quek. Club*, v, ii, 1895, p. 8).

470. CLARKE's **Spirit-proof Cement.**—Mr. CH. ROUSSELET has highly recommended this to me. It may be procured from Mr. J. Bolton, 25, Balshall Heath Road, Birmingham.

ROUSSELET finds it the best he has tried for alcoholic liquids, but not perfectly proof against watery media.

471. Asphalt Varnish (*Bitume de Judée*).—Unquestionably one of the best of these media, either as a cement or a varnish, *provided it be procured of good quality.* It can be procured from the opticians.

472. Brunswick Black.—See *early editions*, or BEALE, *How to Work*, etc., p. 49.

473. Gold-Size.—Best obtained from the opticians. It is soluble in oil of turpentine. A good cement, *when of good quality*, and very useful for turning cells.

474. Marine Glue.—Found in commerce. CARPENTER says the best is that known as G K 4. Best obtained from the opticians.

It is soluble in ether, naphtha, or solution of potash. Its use is for attaching glass cells to slides, and for all cases in which it is desired to cement glass to glass. Used warm.

475. Turpentine, Venice Turpentine (Csokor, *Arch. mik. Anat.*, xxi, 1882, p. 353; Parker, *Amer. Mon. Mic. Journ.*, ii, 1881, pp. 229–30).

Parker's directions are as follows: Dissolve true Venice turpentine in enough alcohol so that after solution it will pass readily through a filter, and after filtering place in an evaporating dish, and by means of a sand-bath evaporate down to about three quarters of the quantity originally used. After it has evaporated down to about that much, drop some of the mass into cold water; if on being taken out of the water it is hard and breaks with a vitreous fracture on being struck with the point of a knife, cease evaporation and allow to cool.

Or (Csokor), "common resinous turpentine of commerce" is put in small pieces to melt over a water-bath, then poured into a suitable vessel and allowed to cool. It should form a brittle, dark brown mass, not yielding to the pressure of a finger. It is sometimes useful, in order to attain the right degree of hardness in the cold mass, to add a little resinous oil of turpentine to the melted mass, and then to evaporate for several hours over the water-bath.

This cement is used for closing glycerin mounts; it is applied in the following manner: Square covers are used, and superfluous glycerin is cleaned away from the edges in the usual way.

The cement is then put on with a piece of wire bent at right angles; the short arm of the wire should be just the length of the side of the cover-glass. The wire is heated in a spirit lamp, plunged into the cement, some of which adheres to it, and then brought down flat upon the slide at the margin of the cover. The turpentine distributes itself evenly along the side of the cover, and hardens immediately, so that the slide may be cleaned as soon as the four sides are finished. It is claimed for this cement that it is perfectly secure, very handy, and never runs in. The cement sets hard in a few seconds.

476. Colophonium and Wax (Krönig, *Arch. mik. Anat.*, 1886, p. 657).—Seven to nine parts of colophonium are added piecemeal to two parts of melted wax, the whole filtered and left to cool. For use, the mass is melted by placing the containing vessel in hot water. The cement is not attacked by water, glycerin, or caustic potash.

477. Apáthy's **Cement for Glycerin Mounts** (*Zeit. wiss. Mik.*, vi, 1889, p. 171).—Equal parts of hard (60° C. melting-point) paraffin and Canada balsam. Heat together in a porcelain capsule until the mass takes on a golden tint and no longer emits vapours of turpentine. On cooling, this forms a hard mass, which is used by warming and applying with a glass rod or brass spatula. One application is enough. The cement does not run in, and never cracks.

478. Paraffin.—*Temporary* mounts may be closed with pure paraffin, by applying it with a bent wire, as described § 475.

479. Canada Balsam, or Damar.—Cells are sometimes made with these. They are elegant, but in my experience are not reliable for permanent mounts.

480. Amber Varnish.—As mentioned § 463, Behrens finds this cement to possess an extreme tenacity. That used by him may be obtained from Grübler & Hollborn.

481. Amber and Copal Varnish (Heydenreich, *Zeit. wiss. Mik.*, 1885, p. 338).—Extremely complicated; may be obtained from Ludwig Marx, at 110, Moskowskaja Sastawa, St. Petersburg; or 79, Gaden, Vienna; or 1, Römerthal, Mayence.

482. Shellac Varnish (Beale, p. 28).—Shellac should be broken into small pieces, placed in a bottle with spirit of wine, and frequently shaken until a thick solution is obtained. The *Micro. Dictionary* says that the addition of 20 drops of castor oil to the ounce is an improvement.

Untrustworthy, but useful for protecting balsam mounts from the action of oil of cedar.

483. Sealing-Wax Varnish (*Micro. Dict.*, "Cements").—Add enough spirit of wine to cover coarsely powdered sealing-wax, and digest at a gentle heat. This should only be used as a varnish, never as a cement, as it is apt to become brittle and to lose its hold upon glass after a time.

484. Tolu Balsam Cement (Carnoy's *Biol. Cell.*, p. 129).

Tolu balsam	2 parts.
Canada balsam	1 part.
Saturated solution of shellac in chloroform	2 parts.

Add enough chloroform to bring the mixture to a syrupy consistence. Carnoy finds this cement superior to all others.

PART II.

SPECIAL METHODS AND EXAMPLES.

CHAPTER XXI.

INJECTION—GELATIN MASSES (WARM).

485. Introduction.—Injection masses are composed of a coloured substance called the *colouring mass,* and of a substance with which that is combined called the *vehicle.*

For instructions as to the operation of injecting, and the necessary apparatus, see the treatises of ROBIN and RANVIER, BEALE'S *How to Work with the Microscope,* the *Lehrbuch der vergleichenden Mikroskopischen Anatomie* of FOL, and (for apparatus especially) the article in the *Encycl. d. mik. Technik.* For injections for the study of the angiology of Vertebrates the practice of Robin and Ranvier may safely be followed. For injections of Invertebrates (and, indeed, for Vertebrates if it is desired to demonstrate the minute structure of environing tissues at the same time as the distribution of vessels) masses not containing gelatin are, I think, generally preferable to gelatin masses; and I would recommend as particularly convenient the Prussian blue glycerin masses of BEALE. Glycerin masses have the great advantage that they are used *cold.*

All formulæ which only give opaque masses, or are only suitable for coarse injections for naked-eye study, have been suppressed.

486. Vaso-dilators.—In order that an injection may run freely it is necessary that the vessels of the subject be in a relaxed state. To this end the older anatomists used to wait until *rigor mortis* had passed off before injecting. But it is evidently preferable in the interest of the proper preservation of the tissues, to inject before *rigor mortis* has set in. Unfortunately, when this is done, it is found that most injection-masses—glycerin masses especially—stimulate the contraction of the vessels, so that frequently it is very difficult to get the injection in. In these cases it may be advisable to use a vaso-dilator. The animal may be anæsthetised with a mixture of ether and *nitrite of amyl,* and finally killed with pure nitrite. Or, after killing by nitrite, a little nitrite of amyl in salt solution may be injected before the injection mass is thrown in. In any case it is advisable to add a little nitrite to the mass just before using. The relaxing power is very great (see OVIATT and SARGENT, in *St. Louis Med. Journ.*, 1886, p. 207; and *Journ Roy. Mic. Soc.*, 1887, p. 341).

Or, *morphia* may be added to the injection-mass, or one per cent. of *lactic acid.* For warm-blooded animals the mass should be warmed to body-temperature; and in all cases masses that tend to dehydrate tissues should be avoided if possible.

ROBIN'S *Masses.*

487. ROBIN'S **Gelatin Vehicle** (*Traité,* p. 30).—Take some good gelatin, soak it in cold water, then heat in water over a water-bath. One part of gelatin should be taken for every 7, 8, 9, or even 10 parts of water; it is a common error to employ solutions containing *too much gelatin.* The solution is now to be combined with one of the colouring masses given below.

This vehicle, like all gelatin masses, is liable to be attacked by mould if kept long; camphor and carbolic acid do not suffice to preserve it.

Chloral hydrate added to the mass is said to preserve it (HOYER). A sufficient dose, at least 2 per cent., should be employed (see § 494).

488. ROBIN'S **Glycerin-gelatin Vehicle** (*Traité,* p. 32).—Dissolve in a water-bath 50 grms. of gelatin in 300 grms. of water, in which has been dissolved some arsenious acid; add of glycerin 150 grams., and of carbolic acid a few drops. Unlike the pure gelatin vehicles, this mass does keep indefinitely.

The colouring masses recommended for combination with the vehicles above described are given in §§ 489 to 491.

FRANKL (*Zeit. F. wiss. Zool.*, lxiii, 1897, p. 28) prepares a similar vehicle, and adds to it a little solution of corrosive sublimate and a crystal of thymol.

489. Robin's **Carmine Colouring Mass** (*Traité,* p. 33).—Rub up in a mortar 3 grms. of carmine with a little water and enough ammonia to dissolve the carmine. Add 50 grms. of glycerin, and filter.

Prepare 50 grms. of acid glycerin (containing 5 grms. of acetic acid for every 50 grms. of glycerin), and add it by degrees to the carmine-glycerin, until a slightly acid reaction is obtained (as tested by very sensitive blue test-paper, moistened and held over the mixture).

One part of this mixture is to be added to 3 or 4 parts of the gelatin injection vehicle (§ 487), or of the glycerin-gelatin (§ 488).

490. Robin's Ferrocyanide of Copper Colouring Mass (*ibid.*, p. 34).—Take—

(1)	Ferrocyanide of potassium (concentrated solution)	20 c.c.
	Glycerin	50 ,,
(2)	Sulphate of copper (concentrated solution)	35 ,,
	Glycerin	50 ,,

Mix (1) and (2) slowly, with agitation; at the moment of injecting combine with 3 volumes of vehicle.

491. Robin's **Prussian Blue Colouring Mass** (*ibid.,* p. 35, and 2nd ed., p. 1013).

Take—

(A)	Ferrocyanide of potassium[*] (sol. sat.)	90 c.c.
	Glycerin	50 ,,
(B)	Liquid perchloride of iron at 30°	3 ,,
	Glycerin	50 ,,

Mix slowly and combine the mixture with 3 parts of vehicle. It is well to add a few drops of HCl.

Carmine-gelatin Masses.

492. Ranvier's **Carmine Gelatine Mass** (*Traité technique,* p. 116).—Take 5 grms. Paris gelatin, soak it in water for half an hour, or until quite swollen and soft; wash it; drain it; put it into a test-tube and melt it, in the water it has absorbed, over a water-bath. When melted add slowly, and with continual agitation, a solution of carmine in ammonia, prepared as follows: 2½ grms. of carmine are rubbed up with a little water, and just enough ammonia, added drop by drop, to dissolve the carmine into a *transparent* solution.

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[*] *Erratim* "Sulphocyanide" in 1st edition of Robin's *Traité.*

When the carmine has been added to the gelatin, you should have about 15 c.c. of ammoniacal solution of carmine in gelatin. This solution is to be kept warm on the water-bath, whilst you proceed to neutralise it by adding cautiously, drop by drop, with continual agitation, a solution of 1 part of glacial acetic acid in two parts of water. (When the mass is near neutrality, dilute the acetic acid still further.) The instant of saturation is determined by the smell of the solution, which gradually changes from ammoniacal to sour. As soon as the sour smell is perceived the liquid must be examined under the microscope. If it contains a granular precipitate of carmine, too much acid has been added, and it must be thrown away.

RANVIER holds that this is the only way to attain to perfect neutralisation. Trust must not be put in formulæ that profess to indicate the proportions of ammonia and acetic acid necessary for neutralisation, on account of the variation in strength of the solutions of ammonia kept in laboratories, and also because it often happens that commercial gelatin is acid.

The mass, having been perfectly neutralised, is strained through new flannel.

493. How to Neutralise a Carmine Mass (VILLE, *Gaz. hebd. d. Sci. méd. de Montpellier*, Fév., 1882; may be had separately from Delahaye et Lecrosnier, Paris).—VILLE points out that when carmine is treated with ammonia a certain proportion of the ammonia combines with the carmine to form a transparent purple compound, and the rest remains in excess. It is this *excess* that it is required to neutralise precisely, not the *whole* of the ammonia employed.

To neutralise the acidity of commercial gelatin, it should be placed in a large funnel with a narrow neck, or better, in a stopcock funnel, and the whole should be placed under a tap, and a stream of water arranged in such a manner that the gelatin be constantly completely immersed. Washing for an hour or so in this way will remove all traces of acids mechanically retained in the gelatin.

As to the neutralisation of the colouring mass, VILLE is of opinion that the sour smell cannot be safely relied on in practice. He considers it greatly preferable to employ exceedingly delicate dichroic litmus paper (litmus paper sensitised so as to be capable of being used equally for the demonstration of acids and bases). Such paper is, I believe, now found in commerce; for directions for preparing it and for preserving ammonia without loss of strength, and other details, see *previous editions.*

494. HOYER'S **Carmine-Gelatin Mass** (*Biol. Centralb.*, 1882, p. 21).—Take a concentrated gelatin solution and add to it the needful quantity of neutral carmine staining solution (*loc. cit.*, p. 17). Digest in a water-bath until the dark violet-red colour begins to pass into a bright red tint. Then add 5–10 per cent. by volumes of glycerin, and at least 2 per cent. by weight of chloral, in a concentrated solution. After passing through flannel it should be kept in an *open* vessel under a bell-glass.

495. FOL'S **Carmine-Gelatin Mass** (*Lehrb.*, p. 13).

This can be *kept in the dry state* for an indefinite length of time. (FOL finds that the addition of chloral hydrate to wet masses is not an efficient preservative.)

Gelatin in sheets is cut into strips which are macerated for two days in carmine solution (prepared by diluting one volume of strong ammonia with three of water and adding carmine *to saturation*, then decanting and allowing to stand for a day or two until the excess of ammonia has evaporated, then filtering). The strips are then rinsed and put for a few hours into water acidulated with acetic acid, then washed on a sieve for several hours in running water, dried on parchment paper, or on a net, and preserved for future use. To get the mass ready for use, the strips are soaked for an hour in water, and melted on a water-bath in 10 to 20 parts of water.

For another process, which is said to give somewhat better results, but is more complicated, see *loc. cit.*, or *Zeit. wiss. Zool.*, xxxviii, p. 492, or *previous editions.*

496. Other Carmine Gelatin Masses.—THIERSCH'S, see *Arch. mik. Anat.*, 1865, p. 148. GERLACH'S, see RANVIER, *Traité*, p. 118. CARTER'S, see Beale, p. 113. Davies, see his *Prep. and Mounting of Mic. Objects*, p. 138.

Blue Gelatin Masses.

497. Robin's **Prussian Blue Gelatin Mass** (see § 491).

498. Ranvier's **Prussian Blue Gelatin Mass** (*Traité*, p. 119). —Twenty-five parts of a concentrated aqueous solution of soluble Prussian blue (prepared as directed next §) mixed with one part of solid gelatin.

The mixture of the Prussian blue with the vehicle is effected in the following manner:

Weigh the gelatin, soak it in water for half an hour or an hour, wash it, and melt it in a test-tube, in the water it has absorbed, by heating over a water-bath. Put the solution of Prussian blue into another test-tube, and heat it on the same water-bath as the gelatin, so as to have the two at the same temperature. Pour the gelatin gradually into the Prussian blue solution, stirring continually with a glass rod. Continue stirring until the disappearance of the curdy precipitate that forms at first. (Some gelatins produce a *persistent* precipitate; these must be rejected; but it must be borne in mind that the precipitate that invariably forms in even the best gelatins disappears if the heating be continued.) As soon as the glass rod has ceased to show blue granulations on its surface on being withdrawn from the liquid, it may be concluded that the Prussian blue is completely dissolved. Filter through new flannel, and keep the filtrate at 40° over a water-bath until injected.

The soluble Prussian blue is prepared as follows:

499. Soluble Prussian Blue (Ranvier, *ibid.*).—Make a concentrated solution of sulphate of peroxide of iron in distilled water, and pour it gradually into a concentrated solution of yellow prussiate of potash. There is produced a precipitate of insoluble Prussian blue. (An excess of prussiate of potash ought to remain in the liquid; in order to ascertain whether this is the case take a small quantity of the liquid and observe whether a drop of sulphate of iron still precipitates it.) Filter the liquid through a felt strainer, underneath which is arranged a paper filter in a glass funnel. The liquid at first runs clear and yellowish into the lower funnel; distilled water is then poured little by little on to the strainer; gradually the liquid issuing from the strainer acquires a blue tinge, which, however, is not visible in that which issues from the lower filter. Distilled water is continually added to the strainer for some days until the liquid begins to run off blue from the second filter. The Prussian blue has now become soluble. The strainer is turned inside out and agitated in distilled water; the Prussian blue will dissolve if the quantity of water be sufficient.

The solution may now be injected just as it is, or it may be kept in bottles till wanted, or evaporated in a stove, and the solid residuum put away in bottle.

For injections, if a simple aqueous solution be taken, it should be *saturated.* Such a mass never transudes through the walls of vessels. Or it may be combined with one fourth of glycerin, or with the gelatin vehicle above described.

500. BRÜCKE'S **Soluble Berlin Blue** (*Arch. mik. Anat.,* 1865, p. 87).

Make a solution of ferrocyanide of potassium containing 217 grammes of the salt to 1 litre of water.

Make a solution of 1 part commercial chloride of iron in 10 parts water.

Take equal volumes of each, and add to each of them twice its volume of a cold saturated solution of sulphate of soda. Pour the chloride solution into the ferrocyanide solution, stirring continually. Wash the precipitate on a filter until soluble, dry it, press between blotting paper in a press, break the mass in pieces, and dry in the air.

The concentrated solution of the colouring matter is to be gelatinised with just so much gelatin that the mass forms a jelly when cold.

For another method, see *previous editions.*

501. Other Blue Gelatin Masses.—HOYER'S, *Arch. mik. Anat.,* 1876, p. 649; GUIGNET'S *Journ. de Microgr.,* 1889, p. 94; *Journ. Roy. Mic. Soc.,* 1889, p. 463; THIERSCH'S, *Arch. mik. Anat.,* i, 1865, p. 148; FOL'S, *Zeit. wiss. Zool.,* xxxviii, 1883, p. 494; and *previous editions.*

Other Colours.

502. HOYER'S **Silver Nitrate Yellow Gelatin Mass** (*Biol. Centralbl.*, ii, 1882, pp. 19, 22).—A concentrated solution of gelatin is mixed with an equal volume of a 4 per cent. solution of nitrate of silver and warmed. To this is added a very small quantity of an aqueous solution of pyrogallic acid, which reduces the silver in a few seconds; chloral and glycerin are added as directed § 494.

This mass is yellow in the capillaries and brown in the larger vessels.

503. Other Colours.—HOYER'S **Green** (*Biol. Centralb.*, ii, 1882, p. 19). Made by mixing a blue mass and a yellow mass. THIERSCH'S **Green** (*Arch. mik. Anat.*, 1865, p. 149). ROBIN'S SCHEELE'S **Green** (ROBIN, *Traité*, p. 37). HARTING'S **White** (see FREY, *Le Microscope*, p. 190). FREY'S **White** (*ibid.*). TEICHMANN'S **White** (*ibid.*, p. 191). FOL'S **Brown** (*Zeit. wiss. Zool.*, xxxviii, 1883, p. 494). MILLER'S **Purple** (see *Amer. Mon. Mic. Journ.*, 1888, p. 50; *Journ. Roy. Mic. Soc.*, 1888, p. 518). FOL'S **Lead Chromate** (*Lehrb.*, p. 15). ROBIN'S **Cadmium** (his *Traité*, p. 36). THIERSCH'S **Lead Chromate** (*Arch. mik. Anat.*, 1865, p. 149). HOYER'S **Lead Chromate** (*ibid.*, 1867, p. 136); or, for any of these, see *early editions.*

504. RANVIER'S **Gelatin Mass for Impregnation** (*Traité*, p. 123).—Concentrated solution of gelatin, 2, 3, or 4 parts; 1 per cent. nitrate of silver solution, 1 part.

505. FRIEDENTHAL'S **Hardening Mass** (*Centralb. Phys.*, xiii, 1899, p. 267).—A 10 per cent. solution of gelatin, combined with a colouring mass, and with 1 vol. of 4 per cent. formol, serves for injecting vessels and hardening the tissues at the same time.

CHAPTER XXII.

INJECTIONS—OTHER MASSES (COLD).

506. FOL'S **Metagelatin Vehicle** (*Lehrb.*, p. 17).—The operation of injecting with the ordinary gelatin masses is greatly complicated by the necessity of injecting them warm. FOL proposes to employ metagelatin instead of gelatin.

If a slight proportion of ammonia be added to a solution of gelatin, and the solution be heated for several hours, the solution passes into the state of metagelatin, that is, a state in which it no longer coagulates on cooling. Colouring masses may be added to this vehicle, which may also be thinned by the addition of weak alcohol. After injection, the preparations are thrown into strong alcohol or chromic acid, which sets the mass.

According to the *Encycl. mik. Technik*, metagelatin is usually prepared by warming with concentrated acetic or oxalic acid. It may be neutralised afterwards with carbonate of lime.

507. TANDLER'S **Cold Gelatin Mass** (*Zeit. wiss. Mik.*, xviii, 1901, p. 22).—Five grms. of gelatin are soaked in 100 c.c. of water, warmed and melted, and combined with Berlin blue. Then five to six grms. of iodide of potassium are slowly incorporated. The mass generally remains liquid enough for injection down to a temperature of 17° C., but if it should coagulate a little more iodide should be added. After injection you may fix with 5 per cent. formol. The specimens will bear decalcification with hydrochloric or sulphurous acid.

Glycerin Masses.

508. BEALE'S **Carmine Glycerin Mass** (*How to Work*, etc., p. 95).—Five grains of carmine are dissolved in a little water with the aid of about five drops of ammonia, and added to half an ounce of glycerin. Then add half an ounce of glycerin with eight or ten drops of acetic or hydrochloric acid, gradually, with agitation. Test with blue litmus paper, and if necessary add more acid till the reaction is decidedly acid. Then add half an ounce of glycerin, two drachms of alcohol, and six drachms of water. I have found this useful, but not so good as the two following.

509. BEALE'S **Prussian Blue** (*How to Work*, etc., p. 93).

Common glycerin	1 ounce.
Spirits of wine	1 ,,
Ferrocyanide of potassium . .	12 grains.
Tincture of perchloride of iron .	1 drachm.
Water	4 ounces.

Dissolve the ferrocyanide in one ounce of the water and glycerin, and add the tincture of iron to another ounce. These solutions should be mixed together *very gradually*, and well shaken in a bottle, *the iron being added to the solution of the ferrocyanide of potassium.* Next the spirit and the rest of water are to be added very gradually, the mixture being constantly shaken.

Injected specimens should be preserved in acidulated glycerine (*e. g.* with 1 per cent. acetic acid), otherwise the colour may fade.

510. BEALE'S **Acid Prussian Blue** (*ibid.*, p. 296).

Price's glycerin	2 fluid ounces.
Tinct. of sesquichloride of iron . .	10 drops.
Ferrocyanide of potassium . . .	3 grains.
Strong hydrochloric acid . . .	3 drops.
Water	1 ounce.

Proceed as before, dissolving the ferrocyanide in one half of the glycerin, the iron in the other, and adding the latter drop by drop to the former. Finally add the water and HCl. Two drachms of alcohol may be added to the whole if desired.

I consider this a most admirable formula. The mass runs well, and has not so much tendency to exude from cut capillaries as might be supposed.

511. RANVIER'S **Prussian Blue Glycerin Mass** (*Traité*, p. 120).—Consists of the Prussian blue fluid, § 499, mixed with one fourth of glycerin.

512. THOMA'S **Indigo-carmine** (*Arch. Anat. Phys., Anat. Abth.*, 1899, p. 270).—Dissolve 0·15 grm. sulphindigotate of soda in 50 c.c. water, filter, add 40 c.c. glycerin and gradually, with agitation, 10 c.c. of a filtered 10 per cent. solution of sodium chloride in water. If desired, 3 c.c. of a 1 per cent. solution of morphia may be added to dilate arteries. A fine precipitate is formed, which is injected with the mass.

513. Gamboge Glycerin (HARTING, *Das Mikroskop*, 1866, 2 Theil, p. 124).—Gamboge rubbed up with water and added to glycerin; or a saturated alcoholic solution of gamboge added to a mixture of equal parts of glycerin and water. Any excess of alcohol may be got rid of by allowing the mass to stand for twenty-four hours.

514. Other Colours.—Any of the colouring masses, §§ 490 to 503, or other suitable colouring masses, combined with glycerin, either dilute or pure.

Purely Aqueous Masses.

515. RANVIER'S **Prussian Blue Aqueous Mass** (*Traité*, p. 120).—The soluble Prussian blue, § 499, injected without any vehicle. It does not extravasate.

516. MÜLLER'S **Berlin Blue** (*Arch. mik. Anat.*, 1865, p. 150).—Precipitate a concentrated solution of Berlin blue by means of ½ to 1 volume of 90 per cent. alcohol.

The precipitate is very finely divided; and the fluid may be injected at once.

517. MAYER'S **Berlin Blue** (*Mitth. Zool. Stat. Neapel*, 1888, p. 307).—A solution of 10 c.c. of tincture of perchloride of iron in 500 c.c. of water is added to a solution of 20 gr. of yellow prussiate of potash in 500 c.c. of water, allowed to stand for twelve hours, decanted, the deposit washed with distilled water on a filter until the washings come through dark blue (one to two days), and the blue dissolved in about a litre of water. It is well to add a little acetic acid, and to put up the objects in an acid liquid.

518. EMERY'S **Aqueous Carmine** (*ibid.*, 1881, p. 21).—To a 10 per cent. ammoniacal solution of carmine is added acetic acid, with continual stirring, until the colour of the solution changes to blood-red through incipient precipitation of the carmine. The supernatant clear solution is poured off, and injected cold without further preparation. The injected organs are thrown at once into strong alcohol to fix the carmine. For injection of fishes.

519. TAGUCHI'S **Indian Ink** (*Arch. mik. Anat.*, 1888, p. 565).—Chinese or (better) Japanese ink well rubbed up on a hone until a fluid is obtained that does not run when dropped on thin blotting-paper, nor form a grey ring round the drop. Inject until the preparation appears quite black, and throw it into some hardening liquid (not pure water).

I believe this will be found useful for work amongst Invertebrates, as well as for lymphatics, juice canals, and the like.

DELLA ROSA (*Verh. Anat. Ges.*, 1900, p. 141) recommends the liquid Chinese ink sold in the shops.

Partially Aqueous Masses.

520. JOSEPH'S **White-of-Egg** (*Ber. naturw. Sect. Schles. Ges.*, 1879, pp. 36—40; *Journ. Roy. Mic. Soc.*, ii, 1882, p. 274).—"Filtered white-of-egg, diluted with 1 to 5 per cent. of carmine solution. . . . This mass remains liquid when cold; it coagulates when immersed in dilute nitric acid, chromic or osmic acid, remains transparent, and is sufficiently indifferent to reagents."

For Invertebrates.

GROSSER (*Zeit. wiss. Mik.*, xvii, 1900, p. 178) rubs up Indian ink with white-of-egg.

521. BJELOUSSOW'S **Gum Arabic Mass** (*Arch. Anat. Phys.*, 1885, p. 379).—Make a syrupy solution of gum arabic and a saturated solution of borax in water. Mix the solutions in such proportions as to have in the mixture 1 part of borax to 2 of gum arabic. Rub up the transparent, almost insoluble mass with distilled water, added little by little, then force it through a fine-grained cloth. Repeat these operations until there is obtained a mass that is free from suspended gelatinous clots. (If the operation has been successful, the mass should coagulate in the presence of alcohol, undergoing at the same time a dilatation to twice its original volume.)

The vehicle thus prepared may be combined with any colouring mass except cadmium and cobalt.

After injection the preparation is thrown into alcohol, and the mass sets immediately, swelling up as above described, and consequently showing vessels largely distended.

Cold-blooded animals may be injected whilst alive with this mass. It does not flow out of cut vessels. Injections keep well in alcohol. Glycerine may be used for making them transparent.

If it be desired to remove the mass from any part of a preparation, this is easily done with dilute acetic acid, which dissolves it.

522. Milk, which was occasionally employed by the old anatomists, has been recently recommended by FISCHER (*Centralb. allg. Path.*, xiii, 1902, p. 277 ; *Zeit. wiss. Mik.*, xx, 1903, p. 224). It runs well, does not extravasate, and can be used for auto-injection of the living subject.

After injection the milk should be coagulated by putting the organs for at least twenty-four hours into a mixture of 75 parts of formol, 15 of acetic acid, and 1000 of water (pure formol will not do). They are then sectioned, and the sections stained with Sudan III or Scharlach R, which stain the mass.

Celloidin and other Masses.

523. SCHIEFFERDECKER'S **Celloidin Masses** (*Arch. Anat. Phys.*, 1882 [*Anat. Abth.*], p. 201). (For Corrosion preparations).—See *previous editions* ; HOCHSTETTER'S **Modification of** SCHIEFFERDECKER'S **Mass** (*Anat. Anz.*, 1886, p. 51) ; BUDGE'S **Asphaltum Mass** (*Arch. mik. Anat.*, xiv, 1877, p. 70), or *early editions* ; HOYER'S **Shellac Mass** (*Arch. mik. Anat.*, 1876, p. 645). For this and that of BELLARMINOW (*Anat. Anz.*, 1888, p. 650), see *early editions* ; HOYER'S **Oil-colour Masses** (*Internat. Monatsschr. Anat.*, 1887, p. 341) ; PANSCH'S **Starch Mass** (*Arch. Anat. Entw.*, 1877, p. 480 ; 1880, pp. 232, 371 ; 1881, p. 76 ; 1882, p. 60 ; 1883, p. 265 ; and a modification of the same by GAGE, *Amer. Mon. Mic. Journ.*, 1888, p. 195) ; TEICHMANN'S **Linseed-Oil Masses** (*S. B. Math. Kl. Krakau Akad.*, vii, pp. 108, 158 ; *Journ. Roy. Mic. Soc.*, 1882, pp. 125 and 716, and 1895, p. 704).

524. Natural Injections (ROBIN, *Traité*, p. 6).—To preserve these throw the organs into a liquid composed of 10 parts of tincture of perchloride of iron and 100 parts of water.

RETTERER and ZENKER use solutions of Müller, see *Journ. Anat. Phys.*, 1894, p. 336, and *Arch. Path. Anat.*, 1894, p. 147.

CHAPTER XXIII.

MACERATION, DIGESTION, AND CORROSION.

Maceration.

525. Methods of Dissociation.—It is sometimes necessary, in order to obtain a complete knowledge of the forms of the elements of a tissue, that the elements be artificially separated from their place in the tissue and separately studied after they have been isolated both from neighbouring elements and from any interstitial cement-substances that may be present in the tissue. Simple teasing with needles is often insufficient, as the cement-substances are frequently tougher than the elements themselves, so that the latter are torn and destroyed in the process. In this case recourse must be had to maceration, by which is meant prolonged soaking (generally for days rather than hours) in media which have the property of dissolving, or at least softening, the cement substances or the elements of the tissue that it is not wished to study, whilst preserving the forms of those it is desired to isolate. When this softening has been effected, the isolation is completed by teasing, or by agitation with liquid in a test-tube, or by the method of tapping, which last gives in many cases (many epithelia, for instance) results which could not be attained in any other way. The macerated tissue is placed on a slide and covered with a thin glass cover supported at the corners on four little feet made of pellets of soft wax. By tapping the cover with a needle it is now gradually pressed down, whilst at the same time the cells of the tissue are segregated by the repeated shocks. When the segregation has proceeded far enough, mounting medium may be added, and the mount closed.

A good material for making *wax feet* is obtained (VOSSELER, *Zeit. wiss. Mik.*, vii, 1891, p. 461) by melting white wax and stirring into it one half to two thirds of Venice turpentine.

The most desirable macerating media are those which, whilst dissolving intercellular substances, do not attack the cells themselves. Those which contain *colloids* have been found to give the best results in this respect (*cf.* § 411). Iodised serum is an example.

526. Iodide of Potassium (ARNOLD, *Arch. mik. Anat.*, lii, 1898, pp. 135 and 763).—10 c.c. of 10 per cent. aqueous sol. of potassic iodide, with 5 to 10 drops of a similar solution, containing also 5 per cent. of iodine.

527. Iodised Serum (§ 415).—The manner of employing it for maceration is as follows: A piece of tissue smaller than a pea must be taken, and placed in 4 or 5 c.c. of weakly iodised serum in a well-closed vessel. After one day's soaking the maceration is generally sufficient, and the preparation may be completed by teasing or pressing out, as indicated § 525; if not, the soaking must be continued, fresh iodine being added as often as the serum becomes pale by the absorption of the iodine by the tissues. By taking this precaution the maceration may be prolonged for several weeks.

These methods are intended to be applied to the preparation of *fresh* tissues, the iodine playing the part of a fixing agent with regard to protoplasm, which it slightly hardens.

528. Alcohol.—RANVIER employs one third alcohol (1 part of 90 per cent. alcohol to 2 parts of water). Epithelia will macerate well in this in twenty-four hours. RANVIER finds it macerates more rapidly than iodised serum.

Other strengths of alcohol may be used, either stronger (equal parts of alcohol and water) or weaker ($\frac{1}{4}$ alcohol, for isolation of the nerve-fibres of the retina, for instance—THIN).

Numerous observers are agreed that one third alcohol is a macerating medium of the highest order.

529. Salt Solution.—10 per cent. solution of sodium chloride is a well-known and valuable macerating medium. Weaker strengths, down to 0·6 per cent., are also used.

529a. MOLESCHOTT and PISO BORME'S **Sodium Chloride and Alcohol** (MOLESCHOTT'S *Untersuchungen zur Naturlehre,* xi, pp.

99—107; Ranvier, *Traité*, p. 242).—10 per cent. solution of sodium chloride, 5 volumes; absolute alcohol, 1 volume.

For vibratile epithelium Ranvier finds the mixture inferior to one third alcohol.

530. Sodium Chloride and Formaldehyde.—Gage recommends the addition of 2 parts of formalin to 1000 parts of normal salt solution. The mixture acts quickly, and yet retards deterioration for some time (quoted from Fish, *Proc. Amer. Mic. Soc.*, xvii, 1895, p. 328).

531. Caustic Potash, Caustic Soda.—These solutions should be employed *strong*, 35 to 50 per cent. (Moleschott); so employed they do not greatly alter the forms of cells, whilst weak solutions destroy all the elements. (Weak solutions may, however, be employed for dissociating the cells of epidermis, hairs, and nails.) The strong solutions may be employed by simply treating the tissues with them on the slide. To make permanent preparations, the alkali should be neutralised by adding acetic acid, which forms with caustic potash acetate of potash, which constitutes a mounting medium (see Behrens, Kossel, and Schiefferdecker, *Das Mikroskop*, i, 1889, p. 156). See also Gage, *Proc. Amer. Soc. of Microscopists*, 1889, p. 35.

532. Baryta-water, Lime-water (Fol, *Lehrb.*, p. 110).—Baryta-water will macerate nerve, muscle, and connective tissue in a few hours, lime-water in a few days.

533. Sulphocyanides of Ammonium and Potassium (Stirling, *Journ. Anat. and Phys.*, xvii, 1883, p. 208).—10 per cent. solution of either of these salts, for epithelium. Macerate small pieces for twenty-four to forty-eight hours.

534. Soulier's Sulphocyanide Mixtures (*Travaux de l'Inst. Zool. de Montpellier*, Nouv. Sér., 2, 1891, p. 171).—Soulier has found that Stirling's solution greatly deteriorates cellular elements, but that good results are obtained by *combining it with a fixing agent.*

The best results were obtained with a 2 per cent. solution of sulphocyanide combined with liquid of Ripart and Petit.

Soulier also obtained good results by combining liquid of Ripart and Petit with artificial serum of Kronecker instead of sulphocyanide, or with pepsin, eau de Javelle, 10 per cent. sulphate of soda, or 1·5 per cent. solution of caustic soda.

And he further found that good results are obtained by combining solutions of chloride of sodium, or solutions of caustic potash or soda, with any of the usual fixing agents.

535. Saliva, Artificial (for embryology of nerve and muscle) (CALBERLA, *Arch. mik. Anat.*, xi, 1875, p. 449).

Second formula (the first is suppressed):

Potassium chloride	0·4
Sodium chloride	0·3
Phosphate of soda	0·2
Calcium chloride	0·2
	1·1

These are dissolved in 100 parts of water, saturated with carbonic acid, and the solution combined with half a volume of MÜLLER's solution and a volume of water.

The MÜLLER's solution may be replaced by a 2½ per cent. solution of chromate of ammonia. The best results were obtained when the solutions were saturated with the CO_2 just before using.

536. LANDOIS's **Solution.** (*ibid.*, 1885, p. 445).

Saturated sol. of neutral chromate of ammonia	5 parts.
Saturated sol. of phosphate of potash	5 ,,
Saturated sol. of sulphate of soda	5 ,,
Distilled water	100 ,,

Small pieces of tissue are macerated for one to three, or even four to five days, in the liquid, then brought for twenty-four hours into ammonia carmine diluted with one volume of the macerating liquid.

GIERKE particularly recommends this liquid for all sorts of macerations, but especially for the central nervous system, for which he finds it superior to all other agents. It is also recommended for the same purpose by NANSEN (v. *Zeit. wiss. Mik.*, v, 1888, p. 242).

537. Bichromate of Potash.—0·2 per cent.

EISIG (*Fauna u. Flora Golf. Neapel*, 16 Monog., 1887, p. 297) macerates Capitellidæ in 0·5 to 1 per cent. solution for months or years, a little thymol being added against mould.

538. Müller's Solution.—Diluted to same strength.

539. Muller's Solution and Saliva (see § 535).

540. Brock's **Medium** (for nervous system of Mollusca, *Intern. Monatssch. Anat.*, i, 1884, p. 349).—Equal parts of 10 per cent. solution of bichromate of potash and visceral fluid of the animal.

541. Permanganate of Potash.—Is recommended, either alone or combined with alum, as the best dissociating agent for the fibres of the cornea (Rollett, *Stricker's Handbuch*, p. 1108). I have found it, for some objects, very energetic.

542. Chromic Acid.—Generally employed of a strength of about 0·02 per cent. Specially useful for nerve tissues and smooth muscle. Twenty-four hours' maceration will suffice for nerve tissue. About 10 c.c. of the solution should be taken for a cube of 5 mm. of the tissue (Ranvier).

543. Osmic and Acetic Acid (the Hertwigs, *Das Nervensystem u. die Sinnesorgane der Medusen*, Leipzig, 1878, and *Jen. Zeitschr.*, xiii, 1879, p. 457).

0·05 per cent.	osmic acid . .	1 part.
0·2 ,,	acetic acid . .	1 ,,

Medusæ are to be treated with this mixture for two or three minutes, according to size, and then washed in repeated changes of 0·1 per cent. acetic acid until all traces of free osmic acid are removed; they then remain for a day in 0·1 per cent. acetic acid, are washed in water, stained in Beale's carmine, and preserved in glycerin.

For *Actiniæ* the osmic acid is taken weaker, 0·04 per cent.; both the solutions are made with sea water; and the washing out is done with 0·2 per cent. acetic acid. If the maceration is complete, stain with picro-carmine; if not, with Beale's carmine.

544. Möbius's **Media** (*Morph. Jahrb.*, xii, 1887, p. 174).

1. One part of sea water with 4 to 6 parts of 0·4 per cent. solution of bichromate of potash.

2. 0·25 per cent. chromic acid, 0·1 per cent. osmic acid, 0·1 per cent. acetic acid, dissolved in sea water. For Lamellibranchiata. Macerate for several days.

545. Nitric Acid.—Most useful for the maceration of muscle. The strength used is 20 per cent. After twenty-four hours' maceration in this, isolated muscle-fibres may generally be obtained by shaking the tissue with water in a test-tube. Preparations may afterwards be washed with water and put up in strong solution of alum, in which they may be preserved for a long time (Hopkins, *Proc. Amer. Soc. of Microscopists*, 1890, p. 165).

Maceration is greatly aided by heat, and at a temperature of 40° to 50° C. may be sufficiently complete in an hour (Gage).

A mixture of equal parts of nitric acid, glycerin, and water is recommended by Marcacci (*Arch. Ital. Biol.*, iv, 1883, p. 293) for smooth muscle.

546. **Nitric Acid and Chlorate of Potash** (Kühne, *Ueber die peripherischen Endorgane*, etc., 1862; Ranvier, *Traité*, p. 79).—Chlorate of potash is mixed, in a watch-glass, with four times its volume of nitric acid. A piece of muscle is buried in the mixture for half an hour, and then agitated with water in a test-tube, by which means it entirely breaks up into isolated fibres.

547. **Nitric and Acetic Acid** (Apáthy, *Zeit. wiss. Mik.*, x, 1898, p. 49).—3 vols. glacial acetic acid, 3 of nitric acid, and 20 each of water, glycerin, and absolute alcohol. Macerate leeches for twenty-four hours, and bring them into 70 per cent. alcohol, in which they swell; then after twenty-four hours, 50 per cent. glycerin, changed till the acid is removed.

548. Béla Haller's **Mixture** (*Morphol. Jahrb.*, xi, p. 321).—One part glacial acetic acid, 1 part glycerin, 2 parts water. Specially recommended for the central nervous system of Mollusca (Rhipidoglossa). A maceration of thirty to forty minutes may be sufficient, the cells showing less shrinkage than with other liquids.

549. **Sulphuric Acid** (Ranvier, *Traité*, p. 78).—Macerate for twenty-four hours in 30 grms. of water, to which are added 4 to 5 drops of concentrated sulphuric acid. Agitate. For nasal mucosa, crystalline, retina, etc.

Odenius found very dilute sulphuric acid to be the best reagent for the study of nerve-endings in tactile hairs. He macerated hair-follicles for from eight to fourteen days in a solution of from 3 to 4 grains of "English sulphuric acid" to the ounce of water.

Hot concentrated sulphuric acid serves to dissociate horny epidermic structures (horn, hair, nails).

550. **Oxalic Acid.**—Maceration for many days in concentrated solution of oxalic acid has been found useful in the study of nerve-endings.

551. SCHIEFFERDECKER'S **Methyl Mixture** (for the retina), (*Arch. mik. Anat.*, xxviii, 1886, p. 305).—Ten parts of glycerin, 1 part of methyl alcohol, and 20 parts of distilled water. Macerate for several days (perfectly fresh tissue).

552. GAGE'S **Picric Alcohol** (*Proc. Amer. Soc. of Microscopists*, 1890, p. 120).—95 per cent. alcohol, 250 parts; water, 750; picric acid, 1. Recommended especially for epithelia and muscle. A few hours' maceration is generally sufficient.

553. **Chloral Hydrate.**—In not too strong solution, from 2 to 5 per cent. for instance, chloral hydrate is a mild macerating agent that admirably preserves delicate elements. LAVDOWSKY (*Arch. mik. Anat.*, 1876, p. 359) recommends it greatly for salivary glands, HICKSON (*Quart. Journ. Mic. Sci.*, 1885, p. 244) for the retina of Arthropods.

554. **Lysol** (REINKE, *Anat. Anz.*, viii, 1892, p. 582).—REINKE uses a 10 per cent. solution in distilled water or in water containing alcohol and glycerin. Spermatozoa of the rat or cortical cells of hairs are said to be resolved into fibrils in a few minutes. Epithelial cells of salamandra are said to be dissociated instantaneously.

Digestion.

555. **Digestion** is maceration in organic juices, which by dissolving out some of the constituents of tissues earlier than others serves to isolate those which resist. The chief

organic liquids employed are gastric juice (or pepsin) and pancreatic juice (pancreatin or trypsin).

Pepsin is best employed in acidified solution, pancreatin in alkaline.

The most favourable temperature for digestion is about 40° C.

Pepsin digests albuminoids, collagen substance and mucin, more or less readily, elastin more slowly. Nuclein is either not dissolved or very slowly. Keratin, neurokeratin, chitin, fat and carbohydrates are not attacked.

Pancreatin (trypsin) digests albuminoids, nuclein, mucin, and elastic tissue; whilst collagen substance, reticular tissue, chitin, horny substances, fat and carbohydrates are not attacked.

Tissues for digestion should be fresh, or fixed with alcohol, not with chromic acid or other salts of the heavy metals.

See on this subject the *Encycl. mik. Technik*, p. 1320.

556. Beale's **Digestion Fluid** (*Archives of Medicine*, i, 1858, pp. 296—316).—The mucus expressed from the stomach glands of the pig is rapidly dried on glass plates, powdered, and kept in stoppered bottles. It retains its properties for years. Eight tenths of a grain will dissolve 100 grains of coagulated white of egg.

To prepare the digestion fluid, the powder is dissolved in distilled water, and the solution filtered. Or the powder may be dissolved in glycerin. The tissues to be digested may be kept for some hours in the liquid at a temperature of 100° F. (37° C.).

557. Brücke's **Digestion Fluid** (from Carnoy's *Biologie cellulaire*, p. 94).

Glycerinated extract of pig's stomach	1 vol.
0·2 per cent. solution of HCl	3 vols.
Thymol, a few crystals.	

558. Bickfalvi's **Digestion Fluid** (*Centrabl. med. Wiss.*, 1883, p. 838).—One grm. of dried stomachal muscosa is mixed with 20 c.c. of 0·5 per cent. hydrochloric acid, and put into an incubator for three or four hours, then filtered. Macerate the tissue in the solution for not more than half an hour to an hour.

559. Kuskow's **Digestion Fluid** (*Arch. mik. Anat.*, xxx, p. 32).—One part of pepsin dissolved in 200 parts of 3 per cent. solution of oxalic acid. The solution should be freshly prepared, and the objects (sections of hardened Ligamentum Nuchæ) remain in it at the ordinary temperature for ten to forty minutes.

560. Schiefferdecker's **Pancreatin Digestion Fluid** (*Zeit. wiss. Mik.*, iii, 1886, p. 483).—A saturated solution of the "Pankreatinum siccum," prepared by Dr. Witte, Rostock, is made in distilled water, cold, and filtered. Pieces of tissue (epidermis) are macerated in it for three to four hours at about body temperature.

561. Kühne's **Trypsin Methods** (see *Unters. a. d. Phys. Inst. Univ. Heidelberg*, i, 2, 1877, p. 219).—Very complicated.

562. Gedoelst's **Methods** (see *La Cellule*, iii, 1887, p. 117, and v, 1889, p. 126).

Corrosion.

563. Corrosion is the operation of destroying the soft parts that surround hard parts that it is desired to study; in short, a means of cleansing hard parts for microscopic study. It has been applied to the removal of surrounding tissue from injected vessels or cavities. For this, see Altmann's Method (*Arch. mik. Anat.*, 1879, p. 471, or *previous editions*; also Rejsek (*Bibliogr. Anat.*, iv, 1897, p. 229); Brühl (*Anat. Anz.*, xiv, 1898, p. 418); Denker (*Anat. Hefte.*, 1900, p. 300); Thoma and Fromherz (*Arch. Entwickelungsmech*, vii, 1898, p. 678); Peabody (*Z. Bull.*, Boston, 1897, p. 164). The following sections relate chiefly to the cleansing of native hard parts.

564. Caustic Potash, Caustic Soda, Nitric Acid.—Boiling, or long soaking in a strong solution of either of these is an efficient means of removing soft parts from skeletal structures (appendages of Arthropods, spicula of sponges, etc.).

565. Eau de Javelle (Hypochlorite of Potash) (NOLL, *Zool. Anzeig.*, 122, 1882, p. 528).—The usual method of preparing the skeleton of siliceous sponges and similar structures by means of caustic potash has many disadvantages, of which a principal one is that the spicula are not preserved in their normal positions. NOLL proceeds as follows: A piece of sponge is brought on to a slide and treated with a few drops of eau de Javelle, in which it remains until all soft parts are dissolved. (With thin pieces this happens in twenty to thirty minutes.) The preparation is then cautiously treated with acetic acid, which removes all precipitates that may have formed, and treated with successive alcohols and oil of cloves, and finally mounted in balsam.

The same process is stated to be applicable to calcareous structures.

566. Eau de Labarraque (Hypochlorite of Soda) may be used in the same way as eau de Javelle. LOOSS (*Zool. Anzeig.*, 1885, p. 333) finds that either of these solutions will completely dissolve chitin in a short time with the aid of heat. For this purpose the commercial solution should be taken concentrated and boiling (see also § 588).

If solutions diluted with 4 to 6 volumes of water be taken, and chitinous structures be macerated in them for twenty-four hours or more, according to size, the chitin is not dissolved, but becomes transparent, soft, and permeable to staining fluids, aqueous as well as alcoholic. The most delicate structures, such as nerve-endings, are stated not to be injured by the treatment. The method is applicable to Nematodes and their ova (objects well known for the resistance they oppose to ordinary reagents), and also to the removal of the albumen from ova of Amphibia, etc.

CHAPTER XXIV.

DECALCIFICATION, DESILICIFICATION, AND BLEACHING.

Decalcification.

567. Decalcification.—In order to obtain the best results, it is important to employ only material that has been *duly fixed and hardened*, and it is well not to put too much confidence in reagents that are said to have the property of hardening and decalcifying fresh material at the same time.

It is generally well also to employ fluids that contain substances having a shrinking action on tissues, so as to neutralise the swelling frequently brought about by the decalcifying acids. Large quantities of liquid should be employed.

After decalcification the excess of acid should be carefully removed by washing, not in water, which favours swelling, but in some liquid that has rather a shrinking action, *e. g.* alum solution. Lastly, the tissues should be neutralised by treatment with carbonate of lime, or a salt of lithium or sodium or the like.

Rousseau (*Zeit. wiss. Mik.*, xiv, 1897, p. 207) imbeds fixed material in celloidin, brings it into 85 per cent. alcohol, decalcifies in a very acid mixture (15 to 40 per cent. of nitric acid in alcohol), washes out the acid in alcohol containing precipitated carbonate of lime, then cuts sections. This for Porifera, corals, Echinoderms, etc. Tissues are said to be well preserved.

This process has been applied to the study of the temporal bone of Mammals by Stein (*Anat. Anz.*, xvii, 1900, p. 318).

568. Decalcification of Bone.—I take the following from Busch, *Arch. mik. Anat.*, xiv, 1877, p. 481; see also Haug, in *Zeit. wiss. Mik.*, viii, 1891, p. 1; and Schaffer, *ibid*, xix, 1903, pp. 308 and 441, and his paper in the *Encycl. mik. Technik.*

The most widely used, though not the best, agent for decalcification is *hydrochloric* acid. Its action is rapid, even when very dilute, but causes serious swelling of the tissues. To remedy this, chromic acid or alcohol may be added to it. Or a 3 per cent. solution of the acid may be taken and have dissolved in it 10 to 15 per cent. of common salt. Or (Waldeyer) to a $\frac{1}{1000}$ per cent. solution of *chloride of palladium* may be added $\frac{1}{10}$ of its volume of HCl.

Chromic acid is also much used, but has a very weak decalcifying action and a strong shrinking action on tissues. For this latter reason it should never be used in solutions of more than 1 per cent. strength, and for delicate structures much lower strengths must be taken.

Phosphoric acid has been recommended for young bones.

Acetic, lactic, and *pyroligneous acids* have considerable decalcifying power, but cause great swelling. *Picric acid* has a very slow action, and is only suitable for very small structures.

569. Nitric Acid (Busch, *loc. cit.*).—To all other agents Busch prefers nitric acid, which causes no swelling and acts most efficaciously.

One volume of chemically pure nitric acid of sp. gr. 1·25 is diluted with 10 vols. water. It may be used of this strength for very large and tough bones; for young bones it may be diluted down to 1 per cent.

Fresh bones are first laid for three days in 95 per cent. alcohol; they are then placed in the nitric acid, *which is changed daily,* for eight or ten days. They must be *removed as soon as* the decalcification is complete, or else they will become stained yellow. When removed they are washed for one or two hours in running water and placed in 95 per cent. alcohol. This is changed after a few days for fresh alcohol.

Young and fœtal bones may be placed in the first instance in a mixture containing 1 per cent. bichromate of potash and $\frac{1}{10}$ per cent. chromic acid, and decalcified with nitric acid of 1 to 2 per cent., to which may be added a small quantity of chromic acid ($\frac{1}{10}$ per cent.) or bichromate of potash (1 per cent.). By putting them afterwards into alcohol the well-known green stain is obtained.

570. Nitric Acid (Schaffer, *Zeit. wiss. Mik.*, xix, 1903, p. 460).—Schaffer also finds nitric acid the best reagent. It should be taken pure; the addition of formol, alcohol, or the like, slows the reaction. The best strength is from 3 to 5 per cent. Objects must not be washed out directly with water, and washing in salt solution, alcohol, phloroglucin, or formol is not sufficient to prevent swelling. Alum in 5 per cent. solution is good, but not necessary. Material should be well fixed and imbedded in celloidin (§ 567); harden in alcohol; remove the alcohol with water; put for 12 to 24 hours (large specimens longer) into nitric acid of 3 to 5 per cent., then into a 5 per cent. solution of sulphate of lithium or sodium, to be changed once in the course of 12 to 24 hours; running water, 48 hours; alcohol.

571. Nitric Acid and Alcohol.—3 per cent. of nitric acid in 70 per cent. alcohol. Soak specimens for several days or weeks. I do not know who first recommended this medium (Mayer has long used 5 per cent. acid in 90 per cent. alcohol). Pure nitric acid, even if weak, readily exercises a gelatinising action on bone; whilst the addition of alcohol (or of *alum*) counteracts this action (Fish, *Ref. Handb. Med. Sci.*, Supp., p. 425).

Thoma (*Zeit. wiss. Mik.*, viii, 2, 1891, p. 191) takes five vols. of 95 per cent. alcohol and 1 volume pure concentrated nitric acid. Leave bones in this mixture, changing the liquid every two or three days, until thoroughly decalcified, which should happen, even with large bones, in two or three weeks. Wash out until every trace of acid is removed (*i. e.* for some days after no acid reaction is obtained with litmus paper) in 95 per cent. alcohol containing an excess of precipitated carbonate of lime. This may take eight to fourteen days, after which the tissues will stain well and may be treated as desired.

572. Nitric Acid and Alum (Gage, quoted from Fish, *loc. cit.* last §).—A saturated aqueous solution of alum is diluted with an equal volume of water, and to each 100 c.c. of the dilute solution is added 5 c.c. of strong nitric acid. Change every two or three days, until the decalcification is complete. For teeth this is said to be, perhaps, a better decalcifier than the alcohol mixture.

573. Sulphurous Acid (ZIEGLER, *Festschr. f. Kupffer,* 1899, p. 51).—A saturated solution in water. Wash out for 24 hours. Acts rapidly and preserves well. Best used after fixation with formol.

574. Hydrochloric Acid (see § 568).—RANVIER says that it may be taken of 50 per cent. strength, and then has a very rapid action. To counteract the swelling action of the acid, sodium chloride may be added (VON EBNER), see HAUG's paper quoted § 568. He takes either 100 c.c. cold saturated solution of sodium chloride in water, 100 c.c. water, and 4 c.c. hydrochloric acid. Preparations to be placed in this, and 1 to 2 c.c. hydrochloric acid added daily until they are soft. Or, 2·5 parts of hydrochloric acid, 500 of alcohol, 100 of water, and 2·5 of sodium chloride. HAUG prefers the proportions of 1·0 to 5·0 of acid, 70 of alcohol, 30 of water, and 0·5 of salt.

575. Hydrochloric Acid and Chromic Acid (BAYERL, *Arch. mik. Anat.*, 1885, p. 35).—Equal parts of 3 per cent. chromic acid and 1 per cent. hydrochloric acid. For ossifying cartilage. HAUG recommends equal parts of 1 per cent. hydrochloric acid and 1 per cent. chromic acid (*loc. cit.*).

576. Hydrochloric Acid and Glycerin.—Glycerin, 95; hydrochloric acid, 5 (SQUIRE's *Methods and Formulæ*, p. 12).

577. Trichloracetic Acid.—PARTSCH (*Verh. Ges. D. Naturf. Aertze,* 1895, 2 *Theil,* 2 Hälfte, p. 26) uses a 5 per cent. aqueous solution, and NEUBERGER (*Centralb. Phys.*, xi, 1897, p. 494) a 4 per cent. one. Action energetic, preservation said to be excellent.

578. Picric Acid should be taken saturated and changed frequently. Its action is weak, but it gives good results with small objects.

Picro-sulphuric acid should, of course, be avoided, on account of the formation of gypsum.

Picro-nitric or Picro-hydrochloric Acid.—Action very rapid.

579. Phosphoric Acid.—10 to 15 per cent. (HAUG, *loc. cit.* in § 568). Somewhat slow, staining not good. According to SCHAFFER, § 570, it produces swelling.

580. Lactic Acid.—10 per cent. or more. Fairly rapid, preserves well, and may be recommended (HAUG, *loc. cit.*).

581. Chromic Acid is employed in strengths of from 0·1 per cent. to 2 per cent. (but see § 568), the maceration lasting two or three weeks (in the case of bone). It is better to take the acid weak at first, and increase the strength gradually. In any way the action is extremely slow, and it is therefore better to take one of the mixtures of chromic acid with a more energetic agent.

582. Chromic and Nitric Acid.—SEILER (FOL, *Lehrb.*, p. 112) takes 70 volumes of 1 per cent. chromic acid, 3 of nitric acid, and 200 of water. Even with the addition of nitric or hydrochloric acid the action is excessively slow, frequently requiring months to be complete.

583. Chromo-aceto-osmic Acid (VAN VER STRICHT, *Arch. Biol.*, ix, 1889, p. 29; and SCHAFFER, *Zeit. wiss. Mik.*, x, 1893, p. 179).—Objects to be left in it for months, the liquid being changed at first every two days, afterwards less frequently. Structure well preserved.

584. Arsenic Acid.—4 per cent. aqueous solution, used at a temperature of 30° to 40° C. (SQUIRE'S *Methods and Formulæ, etc.*, p. 11).

585. Phloroglucin with Acids (ANDEER, *Centralb. med. Wiss.*, xii, xxxiii, pp. 193, 579; *Intern. Monatsschr.*, i, p. 350; HAUG, *Zeit. wiss. Mik.*, viii, 1891, p. 8; FERRERI, *ibid.*, ix, 1892, p. 236; *Bull. R. Accad. Med. di Roma*, 1892, p. 67).—This is the most rapid method of any. Phloroglucin by itself is not a solvent of lime salts; its function in the mixtures given below is so to protect the organic elements of tissues against the action of the mineral acids that these can be used in a much more concentrated form than would be otherwise advisable.

ANDEER takes a saturated solution in warm water, and adds to it 5 to 50 per cent. of hydrochloric acid. Wash out in running water.

HAUG advises the following procedure: Bring one grm. of phloroglucin into 10 c.c. of pure, not fuming, nitric acid (1·4 sp. gr.), and warm very slowly and carefully with gentle agitation. There is formed a clear solution of (presumably) a nitrate of phloroglucin. Dilute the solution with 100 c.c. of distilled water, and add 10 c.c. of nitric acid. This gives a solution containing 20 per cent. of acid, which is the proper proportion. More water may be added to the solution to make it up to

300 c.c. if nitric acid be also added in the proportion given. The process of decalcification in this solution is extremely rapid, and therefore should be carefully watched. Fœtal and young bones become quite soft in half an hour; small pieces of old and hard bones (femur, temporal bone) in a few hours. Teeth take longer, and may require, if time be an object, a solution made with 35 to 45 per cent. of nitric acid. Wash out for two days in running water. The tissues stain well.

The solution may be made with hydrochloric acid instead of nitric acid, 30 per cent. of acid being taken, and 0·5 per cent. of sodium chloride added.

For slow decalcification a 2 to 5 per cent. nitric acid solution may be used, or a mixture containing of phloroglucin 1 part, nitric acid 5, alcohol 70, and distilled water 30 parts.

For the labyrinth, FERRERI advises a mixture containing 1 grm. of phloroglucin, dissolved with the aid of heat in 10 grms. of hydrochloric acid with 100 of water, 200 of 70 per cent. alcohol being added after cooling. The mixture should be changed once a week during thirty to forty days.

Desilicification.

586. Hydrofluoric Acid (MAYER, *Zool. Anz.*, 1881, p. 593).—The objects are brought in alcohol into a glass vessel coated internally with paraffin (otherwise the glass would be corroded by the acid). Hydrofluoric acid is then added drop by drop (the operator taking great care to avoid the fumes, which attack mucous membranes with great energy). Small pieces of siliceous sponges will be completely desilicified in a few hours, or at most a day. The tissues do not suffer.

For sponges I find that this dangerous method can be avoided. If well imbedded, sections may be made from them without previous removal of the spicula, which appear to break off sharp before the knife.

ROUSSEAU imbeds the objects in celloidin, as described § 567, then brings the block, in a covered caoutchouc dish, for a day or two into a mixture of 50 c.c. alcohol and 20 to 30 drops of hydrofluoric acid, and washes out the acid with alcohol containing carbonate of lithia in powder.

Bleaching.

587. MAYER'S **Chlorine Method** (*Mitth. Zool. Stat. Neapel*, ii, 1881, p. 8).—Put into a glass tube a few crystals of chlorate of potash, add two or three drops of hydrochloric acid, and as soon as the green colour of the evolving chlorine has begun to show itself, add a few cubic centimetres of alcohol of 50 to 70 per cent. Now put the objects (which must have previously been soaked in alcohol of 70 to 90 per cent.) into the tube. They float at first, but eventually sink. They will be found bleached in from a quarter of an hour to one or two days, without the tissues having suffered. Only in obstinate cases should the liquid be warmed or more acid taken. Sections on slides may be bleached in this way. Instead of hydrochloric acid nitric acid may be taken, in which case the active agent evolved is oxygen instead of chlorine.

This method serves both for removing natural pigments, such as those of the skin or of the eyes of Arthropods, and also for bleaching material that has been blackened by osmic acid, and, according to renewed experiments of Mayer's, is to be preferred to the peroxide of hydrogen method.

For bleaching chitin of insects, not alcohol but water should be added to the chlorate and acid (Mayer), *Arch. Anat. Phys.*, 1874, p. 321).

588. Eau de Labarraque. Eau de Javelle (see §§ 565, 566).—These are bleaching agents. For the manner of preparing a similar solution see *early editions*, or *Journ. de Microgr.*, 1887, p. 154, or *Journ. Roy. Mic. Soc.*, 1887, p. 518. Of course, the method cannot be used for bleaching soft parts which it is desired to preserve.

589. Peroxide of Hydrogen (Oxygenated Water) (Pouchet's method, M. Duval, *Précis*, etc., p. 234).—Macerate in glycerin, to which has been added a little oxygenated water (§ 38), 5 to 6 drops to a watch-glass of glycerin. Solger (*Centralbl. med. Wiss.*, xxi, 1883, p. 177) takes a 3 per cent. solution of peroxide. Fürst (*Morph. Arb. Schwalbe*, vi, 1896, p. 529) points out that after a time it macerates.

The method serves both for removing pigments and for bleaching osmic and chromic material, see § 38.

590. Peroxide of Sodium (Carazzi, *Zool. Anz.*, 444, 1894, p. 135).—See *previous editions*.

591. Peroxide of Magnesium (Mayer, *Grundzüge*, p. 290).—Use as chloride, § 587. A slow but delicate method.

592. Sulphurous Acid.—Prof. Gilson writes me that he finds alcoholic solution of sulphurous anhydride (SO_2) very convenient for the rapid decoloration of bichromate objects. A few drops suffice. MÖNCKEBERG and BETHE (*Arch. mik. Anat.*, liv. 1899, p. 135) obtain the acid by adding to 10 c.c. of a 2 per cent. solution of bisulphite of sodium 2 to 4 drops of concentrated hydrochloric acid. Objects are put into the freshly prepared solution for six to twelve hours.

593. Permanganate of Potash.—ALFIERI (*Monitore Zool. Ital.*, viii, 1897, p. 57) bleaches celloidin sections of the choroid, etc., for eight to twenty-four hours in a 1 : 2000 solution of permanganate of potash, then washes them out for a few hours in a solution of oxalic acid of 1 : 300 strength, or weaker.

594. GRENACHER'S Mixture for Eyes of Arthropods and other Animals (*Abh. nat. Ges. Halle-a.-S.*, xvi; *Zeit. wiss. Mik.*, 1885, p. 244).

Glycerin	1 part.
80 per cent. alcohol	2 parts.

Mix and add 2 to 3 per cent. of hydrochloric acid.

Pigments [*i. e.* those in question] dissolve in this fluid, and so doing form a stain which suffices in twelve to twenty-four hours for staining the nuclei of the preparation.

595. Nitric Acid.—PARKER (*Bull. Mus. Comp. Zool.*, Cambridge, U.S.A., 1889, p. 173) says that for eyes of scorpions the usual 5 to 10 per cent. solutions are not strong enough. He treats sections, fixed to the slide with SCHÄLLIBAUM'S medium, for about a minute with a solution of up to 50 per cent. of nitric acid in alcohol, or, still better, with a 35 per cent. solution of a mixture of equal parts of nitric and hydrochloric acid in alcohol. To make the solution, the acid should be poured slowly into the alcohol (not *vice versâ*), and the mixture kept cool.

JANDER (*Zeit. wiss. Mik.*, xv, 1898, p. 163) takes for removal of pigments SEILER'S chromo-nitric acid (§ 582); twelve to forty-eight hours is enough for small objects.

596. Caustic Soda.—RAWITZ (*Leitfaden*, p. 29) dissolves the pigment of the mantle of Lamellibranchia by means of 3 to 9 drops of officinal caustic soda solution added to 15 to 20 c.c. of 96 per cent. alcohol.

CHAPTER XXV.

EMBRYOLOGICAL METHODS.*

597. Artificial Fecundation.—This practice, which affords the readiest means of obtaining the early stages of development of many animals, may be very easily carried out in the case of the Amphibia anura, Teleostea, Cyclostomata, Echinodermata, and many Vermes and Cœlenterata.

In the case of the Amphibia, both the female and the male should be laid open, and the ova should be extracted from the uterus and placed in a watch-glass or dissecting-dish, and treated with water in which the testes, or, better, the vasa deferentia, of the male have been teased.

Females of Teleostea are easily spawned by manipulating the belly with a gentle pressure; and the milt may be obtained from the males in the same way. (It may occasionally be necessary, as in the case of the Stickleback, to kill the male, and dissect out the testes and tease them.) The spermatozoa of fish, especially those of the Salmonidæ, lose their vitality very rapidly in water; it is therefore advisable to add the milt immediately to the spawned ova, then add a little water, and after a few minutes put the whole into a suitable hatching apparatus with running water.

Artificial fecundation of Invertebrates is easily performed in a similar way. It is sometimes possible to perform the operation under the microscope, and so observe the penetration of the spermatozoon and some of the subsequent phenomena, as has been done by FOL, the HERTWIGS, SELENKA, and others for the Echinodermata and other forms.

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* The sections in this chapter treating of *Mammalia*, *Aves*, and *Pisces*, closely follow the *Traité des Méthodes Techniques*, LEE et HENNEGUY, and are due almost entirely to HENNEGUY. The corresponding parts of the *Grundzüge*, LEE and MAYER, are taken from this work, and therefore also due to HENNEGUY, which I regret to observe has not always been understood, though duly pointed out in the Preface to the first edition of the *Grundzüge*.

598. Superficial Examination.—The development of some animals, particularly some Invertebrates, may be to a certain extent followed by observations of the living ova under the microscope. This may usefully be done in the case of various Teleosteans, such as the Stickleback, the Perch, *Macropodus*, and several pelagic forms, and with *Chironomus*, *Asellus aquaticus*, Ascidians, *Planorbis*, many Cœlenterata, etc.

Some ova of Insecta and Arachnida which are completely opaque under normal conditions become transparent if they are placed in a drop of oil; if care be taken to let their surface be simply impregnated with the oil, the normal course of development is not interfered with (Balbiani).

599. Fixation.—Osmic acid, employed either alone or in combination with other reagents, is an excellent fixing agent for small embryos, but not at all a good one for large ones. It causes cellular elements to shrink somewhat, and therefore brings out very clearly the slits that separate germinal layers, and any channels or other cavities that may be in course of formation.

In virtue of its property of blackening fatty matters, myelin amongst them, it is of service in the study of the development of the nervous system.

Chromic acid is indispensable for the study of the external forms of embryos; it brings out elevations and depressions clearly, and preserves admirably the mutual relations of the parts; but it does not always preserve the forms of cells faithfully, and is a hindrance to staining in the mass.

Picric liquids have an action which is the opposite of that of osmic acid; they cause cellular elements to swell somewhat, and thus have a tendency to obliterate spaces that may exist in the tissues. But notwithstanding this defect, the picric compounds, and especially Kleinenberg's picro-sulphuric acid, are amongst the best of embryological fixing agents.

Rabl (*Zeit. wiss. Mik.*, xi, 1894, p. 165) recommends for embryos of Vertebrates, and also for other objects:

Platinic chloride, 1 per cent. solution	1 vol.
Saturated aqueous sublimate solution	1 ,,
Distilled water	2 vols.

This serves for a large number of blastoderms and *young* embryos (Pisces, Amphibia, Aves, Mammalia). *Advanced* embryos of Teleostea ought to be fixed in the *warmed* mixture, in order to avoid rupture of the muscles and shrinkage of the chorda.

Some of his best results were obtained by a *not too prolonged* fixation in a mixture of

Platinic chloride, 1 per cent. solution .	1 vol.
Picric acid, saturated aqueous . .	2 vols.
Distilled water	7 „

RABL's picro-sublimate mixture has been given § 75. It is recommended especially for somewhat advanced embryos, such as embryo chicks from the third or fourth day, and other embryos of a similar size.

BOVERI (*Verh. Phys. Med. Ges. Würzburg*, xxix, 1895, p. 4), in order to imbed and cut together numbers of ova of Echinoderms, wraps them in pieces of sloughed epidermis of *Cryptobranchus* (of course, other Urodela will do). SOBOTTA (*Arch. mik. Anat.*, 1, 1897, p. 31) takes pieces of amnios of Mammalia.

599a. PETER's **Double-stain for Yolk and Tissue**, see § 240.

600. Removal of Albumen.—The thick layers of albumen that surround many ova are a serious obstacle to the penetration of reagents. Directions for removing it are given in some of the special sections. CHILD (*Arch. Entwickelungsmech.*, ix, 1900, p. 587; *Zeit. wiss. Mik.*, xvii, 1900, p. 205) gives the following as of very general applicability. After fixation (in any way except with chromic acid) the ova are brought through graduated alcohols up to that of 80 per cent., in which they are hardened. They are then brought down again through successive alcohols into water acidified lightly with any acid (except chromic acid), and the albumen is found to become transparent and dissolve.

601. Reconstruction of Embryos from Sections.—To facilitate the study of series of sections, recourse may be had to processes of graphic or plastic reconstruction.

In simple cases it may be sufficient to adopt the plan described by SCHAFFER (*Zeit. wiss. Mik.*, vii, 1890, p. 342). Careful outlines of the sections to be reconstructed are drawn on tracing paper with the aid of the camera lucida, superposed, and held up against the light for examination by transparence. VOSMAER (*Anat. Anz.*, xvi, 1899, p. 269) draws on plates of celluloid, and sets them up in a rack for examination. KERR (*Quart. Journ. Mic. Sci.*, xlv, 1902, p. 1) draws on plates of ground glass which he afterwards superposes and makes transparent by oil of cloves run in between them.

A method for simple graphic reconstruction without camera drawings is described by WOODWORTH (*Zeit. wiss. Mik.*, xiv, 1897, p. 15): (1) Draw an axial line of the length of the object multiplied by the magnification employed. (2) Measure with a micrometer the greatest diameter of each section. (3) Plot these diameters down transversely on the axial line at distances corresponding to the thickness of the sections multiplied by the magnification. (4) Join the extremities of these diameters; this will give you an outline of the object. (5) Measure off on each section the nearest and farthest limits (from the margin) of the organs to be filled in, and plot them down on the transverse lines (3), and join the points as before, *i. e.* from section to section; this will give you the outlines of the organs.

This process is best applicable to reconstruction from transverse sections, but it can be applied to reconstruction from sections in any plane if the object can be provided with a plane of definition at right angles to the plane of section. This may be established by cutting off one end of the object, or the like (see also *Orientation*, §§ 149, 173).

To make a simple *plastic* reconstruction, camera drawings (or photographs) of the sections (all made at the same magnification) are pasted on pieces of cardboard of a thickness equal to that of the sections multiplied by the magnification employed. Then the parts of the drawings representing the cavities of the objects are cut out with a knife or fretsaw, cutting through the cardboard; and the pieces of fretwork thus obtained are pasted together.

For more elaborate processes of plastic reconstruction (very complicated and seldom necessary) see BORN, "Die Plattenmodellirmethode," in *Arch. mik. Anat.*, 1883, p. 591, and *Zeit. wiss. Mik.*, v, 1888, p. 433; STRASSER, *ibid.*, iii, 1886, p. 179, and iv, pp. 168 and 330; KASTSCHENKO, *ibid.*, iv, 1887, pp. 235–6 and 353, and v, 1888, p. 173; Schaper (*ibid.*, xiii, 1897, p. 446; Alexander, *ibid.*, p. 334, and xv, 1899, p. 446; Born and Peter, *ibid.*, xv, 1, p. 31; and *Verh. Anat. Ges.*, xiii, 1899, p. 134; Johnston, *Anat. Anz.*, xvi, 1899, p. 261; Fol, *Lehrb.*, p. 35, or *previous editions*; Broman, *Anat. Hefte*, xi, 1899, p. 557; and the article "Plastische Rekonstruction," by Peter, in the *Encycl. mik. Technik.*

Mammalia.

602. Rabbit—Dissection.—For the study of the early stages the ova must be sought for in the *tubæ* a certain number of hours after copulation. The dehiscence of the follicles takes place about ten hours after the first coitus. The *tubæ* and *cornua* having been dissected out should be allowed to cool, and remain until the muscular contractions have ceased. Then, with the aid of fine scissors or a good scalpel, all the folds of the genital duct are carefully freed from their peritoneal investment.

The tubæ are then (if the ova are still within them, which is the case up to the end of the third day after coition) laid out on a long slip of glass, and slit up longitudinally by means of a pair of fine, sharp scissors. By means of needles and forceps the tubal mucosa is spread out so as to smooth out its folds as much as possible, and is carefully looked over with a strong lens or with a lower power of the microscope. When the ova are found, a drop of some "indifferent" liquid is dropped on each, and it is carefully taken up with the point of a scalpel, a cataract needle, or a small pipette. They may be examined in the peritoneal fluid of the mother if the animal has been killed, or in its aqueous humour, or in amniotic liquid, or in blood-serum, or in Kronecker's or other artificial serum.

If you have not been able to find the ova with the lens or the microscope, scrape off the epithelium of the tubal mucosa with a small scalpel, mix it with a little indifferent liquid, and look for the ova under the microscope by transmitted light.

Another method, employed by Kölliker, consists in injecting solution of Müller or weak osmic acid into the oviduct by means of a small syringe, and collecting the liquid that runs out in a series of watch-glasses, in which the ova can very easily be found by the microscope.

The same doe may be made to serve for two observations, at some hours' or days' interval. A longitudinal incision of 8 to 10 centimetres' length is made on the median or a lateral line of the abdomen; an assistant keeps the intestines in their place; a ligature is placed at the base of one of the uterine cornua, beneath the neck, and a second ligature around the mesometrium and mesovarium. The ovary, the tuba, and the cornu of that side are then detached with scissors. The abdomen is then closed by means of a few sutures passing through the muscle-layers and the skin. The animals support the operation perfectly well, and the development of the ova of the opposite side is not in the least interfered with. When it is desired to study these the animal may be killed, or may be subjected to a secondary laparatomy if it be desired to preserve it for ulterior observations.

During the *fourth*, *fifth*, and *sixth* days after copulation the ova of the rabbit are free in the uterine cornua; they are easily visible to the naked eye, and may be extracted by the same manipulations as those of the tubes. After the sixth day they are at rest in the uterus, but have not yet contracted adhesions with the mucosa, so that they can still be extracted whole. At this stage the parts of the cornua where the ova are lodged are easily distinguishable by their peculiar aspect, the ova forming eminences of the size of a pea. The cornua should be cut up transversely into as many segments as there are eminences, care being taken to have the ova in the centre of the segments. You then fix each segment by means of two pins on the bottom of a dissecting dish, with the mesometrial surface downwards and the ovular eminence upwards. The dissecting-dish is then filled up with serum or liquid of MÜLLER, or 0·1 per cent. solution of osmic acid, or KLEINENBERG's picro-sulphuric acid, or nitric acid, or acetate of uranium solution. With a small scalpel a longitudinal incision is made on the surface of the ovular eminence, not passing deeper than the muscular layer; the underlying uterine mucosa is then gently dilacerated with two pairs of small forceps, and the ovum set free in the liquid.

From the moment the ova have become adherent to the uterine mucosa they can no longer be extracted whole. The embryo being always situated on the mesometrial surface, the ovular eminence is opened by a *crucial* incision, and the strip of mucosa to which the embryo remains adherent is fixed with pins on the bottom of the dish. Ed. v. Beneden (see *Arch. de Biol.*, v. fasc. iii, 1885, p. 378) has been able by operating in this way in serum of Kronecker, and keeping the whole at blood temperature, to observe the circulation of the embryo for hours together. (If this be desired to be done, the crucial incision should not be too extended, so as to leave the terminal sinus intact.)

Retterer (*C. R. Soc. de Biol.*, 1887, p. 99) advises that for ova of the seventh day the segment of uterus containing them be opened *on the mesometrial surface*, for at that date no adhesion has yet been contracted with that side. By running in liquid of Kleinenberg by means of a pipette between the ovum and the free surface of the uterus, the ovum may be got away in the shape of a closed vesicle.

603. Rabbit ; **Microscopic Preparations.**—In order to make permanent preparations of the different stages of fecundation and segmentation, v. Beneden (*Arch. de Biol.*, i, 1, 1880, p. 149) brings the living ovum into a drop of 1 per cent. osmic acid on a slide, and thence into solution of Müller (or bichromate of ammonia or solution of Kleinenberg). After an hour the liquid is changed, and the whole is put into a moist chamber, where it remains for two or three days. It is then treated with glycerin of gradually increasing strength, and at last mounted in pure glycerin acidified with formic acid. Ova may be stained after careful washing.

In order to bring out the outlines of blastoderm cells the living ovum may be brought into one third per cent. solution of nitrate of silver. After remaining there for half a minute to two minutes, according to the age of the vesicle, it is brought into pure water and exposed to the light. The preparations thus obtained are instructive, but blacken rapidly, and cannot be permanently preserved.

After the end of the third day the blastodermic vesicle can be opened with fine needles, and the blastoderm washed, stained, and mounted in glycerin or balsam ; v. Beneden has also obtained good preparations by means of chloride of gold.

For embryonic areas and more advanced embryos Kölliker recommends putting the ovum into 0·5 per cent. solution of osmic acid until it has taken on a somewhat dark tint, which happens in about an hour, and then treating it with successive alcohols for several hours. If the ovum be adherent to the uterine mucosa the portion of the membrane to which it is fixed should be left, stretched out with pins, in 0·1 per cent. solution of osmic acid for from four to six hours. The blastodermic vesicle can then easily be removed, and further treated as before. For sections KÖLLIKER fixes with osmic acid. v. BENEDEN treats the ova for twenty-four hours with 1 per cent. solution of chromic acid, then washes well, and brings them through successive alcohols. Chromic acid has the advantage of hardening thoroughly the vesicle, and maintaining at the same time the epiblast cells perfectly adherent to the zona pellucida. v. BENEDEN also recommends the liquid of Kleinenberg. HENNEGUY writes that he frequently employs it for embryonic areas and embryos of various ages, always with excellent results. Fol's modification of the liquid of Flemming, and Ranvier and Vignal's osmic acid and alcohol mixture (§ 40) also give excellent results. For staining, HENNEGUY recommends borax-carmine, or Delafield's hæmatoxylin for small embryos; for large ones he found that his acetic acid alum-carmine was the only reagent that would give a good stain in the mass. I think carmalum is now indicated.

For sections imbed in paraffin.

See also WEYSSE, *Proc. Amer. Acad. Arts and Sci.*, 1894, p. 285 (blastodermic vesicle of *Sus scrofa*); SOBOTTA, *Arch. mik. Anat.*, xlv, 1895, p. 15 (fecundation and segmentation of the ovum of the mouse; fixation in FLEMMING's weak mixture, sections stained with BENDA's iron hæmatoxylin), and *Anat. Hefte*, 1 Abth., viii, 1897, p. 476 (Rabbit; fixation with liquid of Flemming or picro-sublimate with 2 per cent. acetic acid); BONNET, *ibid.*, ix, 1897, p. 426 (Dog; fixation in sublimate); SELENKA, *Stud. Entw. d. Thière*, Wiesbaden, 1883, p. 5, and 1887, p. 107 (picrosulphuric acid for the mouse, and picric acid with $\frac{1}{10}$ per cent of chromic acid for *Didelphys*); KEIBEL, Morph. Arb., ii, 1893, p. 11 (*Sus scrofa*); NEUMAYER, *Festschr. f. Kupffer*, 1899, p. 458 (embryos of the sheep best fixed in Carnoy's acetic acid, alcohol, and chloroform, § 90); WINIWARTER, *Arch. Biol.*, xvii, 1900, p. 39 (mixture of 50 parts saturated sublimate in salt solution, 50 parts alcohol, 20 of 1 per cent. platinum chloride, and 5 of acetic acid).

Aves.

604. Superficial Examination.—Instructions on this head are given in FOSTER and BALFOUR'S *Elements of Embryology*. The following is of more recent publication.

If it be desired to observe a living embryo by transmitted light, the egg should be opened under salt solution, as described below. A little of the white is then removed through the window, the egg is lifted out of the liquid, and a ring of gummed paper is placed on the yolk so as to surround the embryonic area. As soon as the paper adheres to the vitelline membrane, which will be in a few minutes, a circular incision is made in the blastoderm outside the paper ring. The egg is put back into the salt solution, and the paper ring removed, carrying with it the vitelline membrane and the blastoderm, which may then be brought into a watch-glass or on to a slide and examined under the microscope (DUVAL).

605. Gerlach's Window Method (*Nature*, 1886, p. 497).—Remove with scissors the shell from the small end of the egg; take out a little white by means of a pipette; the blastoderm will become placed underneath the window thus made, and the white that has been taken out may be replaced on it. Paint the margins of the window with gum mucilage, and build up on the gum a little circular wall of cotton wool; place on it a small watch glass (or circular cover glass), and ring it with gum. When the gum is dry the cover is further fixed in its place by means of collodion and amber varnish, and the egg is put back in its normal position in the incubator. The progress of the development may be followed up to the fifth day through the window.

A description of further developments of this method, with figures of special apparatus, will be found in *Anat. Anz.*, ii, 1887, pp. 583, 609.

606. Preparation.—During the first twenty-four hours of incubation it is extremely difficult to separate the blastoderm from the yolk, and they should be fixed and hardened together.* In later stages, when the embryo is conspicuous, the blastoderm can easily be separated from the yolk, which is very advantageous. To open the egg, lay it on its side and break the shell at the broad end by means of a sharp rap; then carefully remove the shell bit by bit by breaking it away with forceps, working away from the broad end until the blastoderm is exposed. The egg should be opened in salt solution, then lifted up a little, so as to have the blastoderm above the surface of the liquid; the blastoderm is then treated with some fixing solution dropped on it from a pipette (1 per cent. solution of osmic acid, or Ranvier and Vignal's osmic acid and alcohol mixture, iodised serum, solution of Kleinenberg, 10 per cent. nitric acid, etc.). By keeping the upper end of the pipette closed, and the lower end in contact with the liquid on the blastoderm, the blastoderm may be kept well immersed for a few minutes, and should then be found to be sufficiently fixed to be excised. (Of course, if you prefer it, you can open the egg in a bath of any fixing liquid [10 per cent. nitric acid being convenient for this purpose] of such a depth as to cover the yolk; and having exposed the blastoderm, leave it till fixed [fifteen to twenty minutes]; but I think the procedure above described will generally be found more convenient.)

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* ANDREWS (*Zeit. wiss. Mik.*, xxi, 1904, p. 177) separates the blastoderm at this stage by injecting picro-sulphuric acid (not any rapidly acting fixative) firstly, between the blastoderm and the vitelline membrane, so as to separate the two above, and then between the blastoderm and the yolk, so as to free the blastoderm below and float it up. This done, the membrane may be incised and the blastoderm removed. The injection is best done with a pipette having a fine point bent upwards.

The egg is put back into the salt solution, and a circular incision made round the embryonic area. The blastoderm may then be floated out and got into a watch-glass, in which it may be examined, or may be brought into a hardening liquid.

Before putting it into the hardening fluid, the portion of vitelline membrane that covers the blastoderm should be removed with forceps and shaking.

Fixation in 10 per cent. nitric acid has the advantage of greatly facilitating the separation of the blastoderm. The acid should be allowed to act for ten minutes, after which it is well to bring the preparation into 2 per cent. solution of alum (*cf.* HOFMANN, *Zeit. wiss. Mik.*, x, 1893, p. 485). MITROPHANOW (*Anat. Hefte*, xii, 1899, p. 200) fixes with nitric acid of 3 per cent., SUSCHKIN (*Nouv. Mém. Soc. Nat. Moscow*, xvi, 1899, p. 34) with sublimate; and FISCHEL (*Morph. Jahrb.*, xxiv, 1896, p. 371) with Rabl's platino-sublimate, § 599 (embryos of the duck).

In order to counteract the turning up of the edges of the blastoderm that generally happens during the process of hardening, it is well to get the blastoderm spread out on the *convex* surface of a watch-glass, and leave it so during the hardening.

For hardening HENNEGUY prefers the osmic acid and alcohol mixture of Ranvier and Vignal, or Flemming's mixture followed by successive alcohols.

Stain and imbed by the usual methods.

Up to about the fiftieth hour embryos may be mounted entire in glycerin or balsam.

607. M. DUVAL's **Orientation Method** (*Ann. Sc. Nat.*, 1884, p. 3).—In the early stages of the development of the ova of Aves, before the appearance of the primitive streak, it is difficult to obtain a correct orientation of the hardened cicatricula, so as to be able to make sections in any desired direction. DUVAL, starting from the fact that during incubation the embryo is almost always found to be lying on the yolk in such a position that the big end of the egg is to the left, and the little end to the right of it, marks the position of the blastoderm in the following way.

With a strip of paper 5 millimetres wide and 50 millimetres long you construct a sort of triangular bottomless box. You lay this on the yolk, enclosing the cicatricula in such a position that the base of the triangle corresponds to what will be the anterior region of the embryo, and its apex to the posterior region; that is to say, if the big end of the egg is to your left, the apex of the triangle will point towards you. You now, by means of a pipette, fill the paper triangle with 0·3 per cent. solution of osmic acid. As soon as the preparation begins to darken you put the whole egg into weak chromic acid, remove the white, and put the rest into clean chromic acid solution for several days. After hardening you will find on the surface of the yolk a black triangular area, which encloses the cicatricula and marks its position; you cut out this area with scissors and a scalpel, and complete the hardening with chromic acid and alcohol.

See also the method of HIROTA, *Journ. Roy. Mic. Soc.*, 1895, p. 118.

608. KIONKA's **Orientation Method** (*Anat. Hefte,* 1 Abth., iii. 1894, p. 414).—Open the egg under salt solution, free it from the shell and albumen, and mark the poles by sticking into it, at about a centimetre from the blastoderm, two hedgehog spines, the one at the obtuse end being marked with a red thread. Put the whole for ten minutes into water at 90° C., then bring into 70 per cent. alcohol, and after twenty-four hours cut out the blastoderm and a little yolk round it in the shape of an isosceles triangle, whose base marks the anterior end of the blastoderm. Paraffin sections stained with borax-carmine, washed out with acid alcohol containing one drop of concentrated solution of Orange G for each 5 c.c., which stains the yolk.

609. Vialleton's **Method** (*Anat. Anz.*, vii, 1892, p. 624).—Egg opened in salt solution, blastoderm excised and removed to a glass plate, then treated with 1 per cent. nitrate of silver solution, washed with water, and put into 70 per cent. alcohol for six to twelve hours in the dark. Borax-carmine, alcohol, damar.

610. Böhm and Oppel (*Taschenbuch*, 1896, p. 80) fix ova with fairly large embryos in a mixture of 20 parts 3 to 5 per cent. nitric acid and 1 to 2 parts 1 per cent. silver nitrate.

Reptilia.

611. General Directions.—The methods described above for birds are applicable to reptiles. During the early stages the blastoderm should be hardened *in situ* on the yolk; later the embryo can be isolated, and treated separately with fixatives, etc.

Böhn and Oppel (*Taschenbuch*, 1900, p. 186) remove the shell under salt solution, fix in sublimate with 20 per cent. acetic acid, or in Lo Bianco's chromo-sublimate (§ 77), then remove the blastoderm and bring it into alcohol.

612. Special Cases.—Mitsukuri (*Journ. Coll. Sc. Japan*, vi, 1894, p. 229) fixes embryos of tortoises chiefly with picro-sulphuric acid. To study the blastoderm he removes the whole of the shell and as much as possible of the albumen, marks the place where the blastoderm lies with a hair, brings the whole, with the blastoderm uppermost, into the fixative, and after a few hours cuts out the blastoderm and further hardens it by itself. Young embryos generally adhere to the shell and can, therefore, be fixed in a piece of it made to serve as a watch-glass, then after half an hour can be removed from it and further hardened alone. If the embryonal membranes have been formed, the shell may be scraped away at some spot and there treated with picro-sulphuric acid until a small hole is formed; then by working away from this spot, by means of scraping and dropping acid on to it, the whole of the shell may be removed.

WILL (*Zool. Jahrb., Abth. Morph.*, vi, 1892, p. 8) opens ova of *Platydactylus* in the fixative (chiefly chromic acid, or chromo-aceto-osmic acid with very little osmic acid) and hardens the embryos on the yolk; so also for *Cistudo* and *Lacerta* (1893 and 1895). MEHNERT (*Anat. Anz.*, xi, 1895, p. 257) does not approve of these methods; for his own see *Morph. Arb. Schwalbe*, i, 1891, p. 370.

STRAHL (*Arch. Anat. Phys., Anat. Abth.*, 1881, p. 123) uses picro-sulphuric acid for *Lacerta*.

KUPFFER (*op. cit.*, 1882, p. 4) removes ova of *Lacerta, Emys, Coluber*, etc., from the oviduct, opens them under 0·1 per cent. osmic acid, removes as much of the albumen as possible, brings the yolk for twenty-four hours into chromic acid of 1 : 300 strength, then excises the blastoderm, washes, puts it for three hours into a mixture of glycerin, alcohol, and water in equal parts, and lastly into 90 per cent. alcohol.

NICOLAS (*Arch. Anat. Mic.*, 1900, p. 457) finds the best fixative for ova of the slow-worm, as for other large ova is BOUIN'S picro-formol (§ 117).

See also PERENYI, § 52, and *Zool. Anz.*, 1888, pp. 139 and 196, and other methods in *early editions*.

Amphibia.

613. Preliminary.—In order to prepare ova for section-cutting, it is essential to begin by removing their thick coats of albumen. This may be done by putting them for two or three days into 1 per cent. solution of chromic acid, and shaking well; but ova thus treated are very brittle, and do not afford good sections. A better method is that described by WHITMAN (*Amer. Natural.*, xxii, 1888, p. 857), and by BLOCHMANN (*Zool. Anz.*, 1889, p. 269). WHITMAN puts the fixed eggs into a 10 per cent. solution of sodium hypochlorite diluted with 5 to 6 volumes of water, and leaves them there till they can be shaken free, which happens (for *Necturus*) in a few minutes. Blochmann takes *eau de Javelle* (potassium hypochlorite), and dilutes it with 3 to 4 volumes of water, and agitates the eggs previously fixed with solution of Flemming, for fifteen to thirty minutes in it. See also § 600.

Lebrun (*La Cellule*, xix, 1902, p. 316) advises fixing ova of Anura for not less than 1½ hours in liquid of Gilson, § 74. The outer envelopes are then hard, and may be easily incised and the ovum extracted by pressing on the pole opposite to the incision. The operation should *not* be delayed until after hardening in alcohol. Similarly (*ibid.*, xx, 1902, p. 12), for Urodela.

614. Imbedding.—A great difficulty with the ova of Amphibia lies in their becoming extremely brittle on imbedding in paraffin. Carnoy and Lebrun (*La Cellule*, xii, 1897, p. 212) fix ovaries or ovarian ova for fifteen minutes to three quarters of an hour (but see last §) in Gilson's mercuro-nitric fluid, § 74, and preserve them in 80 per cent. alcohol. To imbed, they are brought for a quarter of an hour into 95 per cent. alcohol, five minutes in absolute alcohol, then into a mixture of alcohol and chloroform in equal parts, and as soon as they sink in that they are put into pure chloroform. Paraffin is added to the chloroform, enough to about double the volume of the whole, and the whole is put for about three hours into a stove at 35° C. Lastly, the ova are put *for not more than five minutes* into a bath of pure paraffin at 52° C.

Later (*ibid.*, xix, 1902, p. 317) Lebrun explains that it is important not to dehydrate completely with absolute alcohol; the ova should be left in alcohol of 96 per cent. until chloroform can be added without the mixture becoming turbid, and a second bath of clean paraffin should be added.

See also Morgan, *Devel. of the Frog's Egg*, New York, 1897, p. 171.

615. Axolotl.—The ova are easier to prepare than those of the Anura, because the yolk is separated from the albuminous layer by a wide space filled with a liquid that is not coagulated by reagents. Put the eggs for a few hours into picro-sulphuric acid, then pierce the inner chorion with fine scissors or needles, and gently press out the ovum. Harden in alcohol.

Fick (*Zeit. wiss. Zool.*, lvi, 1893, p. 529) uses a mixture of 250 parts of 1 per cent. chromic acid, 1 of acetic acid, and 750 of water.

616. Triton (Scott and Osborn, *Quart. Journ. Mic. Soc.*, 1879, p. 449).—The albumen is here present in the form of several concentric coats, which are very delicate. Incise each of them separately with fine scissors, turn out the ovum, and fix it. Solution of Kleinenberg is the reagent that gives the best results.

Hertwig (*Jen. Zeit. Naturw.*, 1881–2, p. 291) puts the eggs into a mixture of equal parts of 2 per cent. acetic acid and 0·5 per cent. chromic acid. After ten hours he incises the membranes, opening one end of the inner chorion, and turns out the embryos and brings them into successive alcohols.

Braus (*Jena Zeit.*, xxix, 1894, p. 443) fixes ova to a piece of liver by passing an entomological pin through the albumen, then incises the albumen and turns out the ova into fixing liquid.

Michaelis (*Arch. mik. Anat.*, xlviii, 1896, p. 528) fixes ova in a mixture of concentrated sublimate solution and concentrated picric acid, twenty parts each, glacial acetic acid 1, and water 40, but removes the envelopes before bringing into alcohol.

617. Salamandra (Rabl, *Morphol. Jahrb.*, xii, 2, 1886, p. 252).—For his more recent methods see § 599.

618. Rana (O. Hertwig, *Jen. Zeit. Naturw.*, xvi, 1883, p. 249).—The ova are thrown into nearly boiling water (90° to 96° C.) for five or ten minutes. The albuminous envelope of the ovum is then cut open, and the ovum extracted under water. The ova are then brought into 0·5 per cent. chromic acid for not more than twelve hours, or into alcohol of 70, 80, and 90 per cent. Chromic acid makes ova brittle and attacks the pigment, whilst alcohol preserves it, which is frequently important for the study of the germinal layers.

Morgan (*Amer. Nat.*, xxv, 1891, p. 759) has the following. During the periods in which it is difficult or impossible to remove the inner jelly-membrane the eggs can be freed as follows: Each egg is cut out with scissors from the general jelly-mass, and put for from one to twelve hours into saturated solution of picric acid in 35 per cent. alcohol containing "the same amount of sulphuric acid as in Kleinenberg's solution." Wash for several hours in several changes of alcohol, beginning with 35 per cent., and increasing the strength gradually up to 70 per cent. About the second day in the 70 per cent. alcohol the inner membrane begins to swell, and on the third or fourth day may be pierced by a needle, and the egg removed and placed in 80 per cent. alcohol (see also WHITMAN, *Meth. of Research*, p. 156).

SCHULTZE (*Arch. mik. Anat.*, lv, 1899, p. 174) removes with scissors the outer layers of albumen, and puts the ova for five minutes in 2 per cent. formol warmed to 75° or 80° C. The membrane left on the ova then rises up sufficiently to allow the ova to be got out with needles.

See also BORN (*ibid.*, xliii, 1894, p. 1).

619. Sulphate of Copper Liquid (FOL, *Lehrbuch*, p. 106, after REMAK and GOETTE); for hardening ova of Amphibia:

2 per cent. solution of sulphate of copper .	50 c.c.
Alcohol of 25 per cent.	50 ,,
Rectified wood vinegar	35 drops.

Pisces.

620. Teleostea in General.—The ova of many of the bony fishes can be studied by transmitted light in the living state; but those of the Salmonidæ must be hardened and removed from their envelopes for the study of the external forms of the embryo.

To this end the ova may be put for a few minutes into water containing 1 to 2 per cent. of acetic acid, and thence into 1 per cent. chromic acid. After three days the capsule of the ovum may be opened at the side opposite to the embryo, and be removed with fine forceps. The ovum is put for twenty-four hours into distilled water, and then into successive alcohols. Embryos thus prepared show no deformation, and their histological elements are fairly well preserved. But the vitellus rapidly becomes excessively hard and brittle, so as greatly to interfere with section-cutting.

The following processes give good results as regards section-cutting.

Put the ova for a few minutes into 1 per cent. osmic acid; as soon as they have taken on a light brown colour bring them into Müller's solution. Open them therein with fine scissors—the vitellus, which immediately coagulates on contact with air, dissolves, on the contrary, in Müller's solution—and the germ and cortical layer can be extracted from the capsule of the ovum. They should be left in clean Müller's solution for a few days, then washed with water for twenty-four hours, and brought through successive alcohols.

Another method (HENNEGUY) is as follows: The ova are fixed in solution of Kleinenberg containing 10 per cent. of acetic acid. After ten minutes they are opened in water containing 10 per cent. of acetic acid, which dissolves the vitellus. The embryos are put for a few hours into pure solution of Kleinenberg, and are then brought through alcohol of gradually increasing strength.

CHILD (quoted from SUMNER, *Mem. New York Acad. Sci.*, ii, 1900, p. 78) fixes for about a minute in sublimate with 10 per cent. of acetic acid, and brings into formalin of 10 per cent., which is said to give a good fixation of the embryo without the yolk becoming hard.

621. KOLLMANN'S **Fixative** (KOLLMANN, *Arch. Anat. Phys.*, 1885, p. 296).

Bichromate of potash	5 per 100.
Chromic acid	2 „
Concentrated nitric acid	2 „

For ova of Teleostea. Fix for twelve hours, wash with water for twelve hours, then remove the chorion, and put the ova into 70 per cent. alcohol.

622. RABL'S **Method** (see § 599).

623. KOWALEWSKY'S **Method** (see *Zeit. wiss. Zool.*, xliii, 1886, p. 434, or *Third Edition*).

624. Salmonidæ.—HENNEGUY'S methods have been given, § 620.

KOPSCH (*Arch. mik. Anat.*, li, 1897, p. 184), on the suggestion of VIRCHOW, fixes the embryos for five or ten minutes in a mixture of 1 part of chromic acid to 50 of glacial acetic acid and 450 of water, then removes them into chromic acid of 1 : 500, and as soon as may be removes the capsule and yolk *under salt solution,* and completes the hardening in the chromic acid or saturated sublimate solution.

Similarly BEHRENS (*Anat. Hefte,* x, 1898, p. 233).—He leaves the ova for about an hour in the chromic acid, not much more anyway; he opens them in the salt solution from the antipolar side, and frees the embryo from the yolk that remains by blowing the latter away with a fine pointed glass tube; after which the embryo can be easily detached from the capsule. It is then removed for about three hours into a mixture of 1 part saturated picric acid solution, 1 part saturated sublimate solution, and 2 parts distilled water, after which it is treated in the usual way with iodine and successive alcohols.

Similarly also SOBOTTA (*ibid.,* 1902, p. 579).

RABL-RÜCKHARD'S Method (*Arch. Anat. Entw.,* 1882, p. 118).—Fix in 10 per cent. nitric acid for fifteen minutes. Remove the membranes to avoid deformation of the embryos, and put the ova back into the acid for an hour. Wash out in 1 to 2 per cent. solution of alum for an hour, and harden in alcohol.

Modification of this method by GORONOWITSCH (see *Morph. Jahrb.,* x, 1884, p. 381).

HARRISON (*Arch. mik. Anat.,* xlvi, 1895, p. 505) fixes in saturated solution of sublimate in 5 per cent. acetic acid.

FELIX (*Anat. Hefte,* 1 Abth., viii, 1897, p. 252) fixes ova for three-quarters of an hour in acetic sublimate, but dissected-out embryos in liquid of Zenker, the vitellus being removed from the abdominal cavity with a brush.

625. Selachia.—BEARD (*Anat. Anz.,* xviii, 1900, p. 556) has found that the best fixatives for embryos of *Raja* are Rabl's picro-platinic mixture, § 599, and sublimate.

626. Amphioxus.—SOBOTTA (*Arch. mik. Anat.,* 1, 1897, p. 20) advises fixing for twenty-four hours in liquid of Flemming; HATSCHEK (*Arb. Zool. Inst. Wien,* iv, 1881) in picro-sulphuric acid. Impregnation takes place in the evening, and segmentation is completed during the night.

627. Pelagic Fish Ova.—WHITMAN (*Amer. Natural,* xvii, 1883, pp. 1204–5; and *Methods of Research,* etc., p. 152).—Fix by treatment first for five to ten minutes with a mixture of equal parts of sea water and ½ per cent. osmic acid solution, and then for one or two days with a

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solution (due to Eisig) of equal parts of 0·25 per cent. platinum chloride and 1 per cent. chromic acid. Prick the membrane before transferring to alcohol. See also Agassiz and Whitman, in *Proc. Amer. Acad. Arts and Sciences*, xx, 1884; and Collinge, *Ann. and Mag. Nat. Hist.*, x, 1892, p. 228.

Raffaele (*Mitth. Zool. Stat. Neapel*, xii, 1895, p. 169) fixes chiefly with liquid of Hermann (1 to 2 days), or with mixture of Mingazzini (absolute alcohol 1, acetic acid 1, saturated sublimate solution in water 2).

Heinke and Ehrenbaum (*Wiss. Meeresunt. Komm. Wiss. Unt. D. Meere*, iii, Heligoland, 1900, pp. 205 and 213) prefer formol with 39 vols. of sea-water.

Tunicata.

628. Ova.—Davidoff (*Mitth. Zool. Stat. Neapel*, ix, 1, 1889, p. 118) fixes the ova of *Distaplia* with a mixture of 3 parts of saturated solution of corrosive sublimate and 1 of glacial acetic acid. The ova to remain in it for from half an hour to an hour, and be then washed for a few minutes in water and brought through successive alcohols. Another reagent, almost as good, consists of 3 parts of saturated solution of picric acid and 1 of glacial acetic acid, the objects to remain in it for three to four hours, and then be brought into 70 per cent. alcohol.

Castle (*Bull. Mus. Harvard Coll.*, xxvii, 1896, p. 213) advises for ova of *Ciona* liquid of Perényi for twenty minutes, followed by 70 per cent. alcohol for twenty-four hours, and for the larvæ picro-nitric acid.

629. Test-Cells of Ascidians (Morgan, *Journ. of Morphol.*, iv, 1890, p. 195).—Tease fresh ovaries in very weak osmic acid, wash in distilled water, treat for half an hour with 1 per cent. silver nitrate, wash for half an hour in 2 per cent. acetic acid, and reduce in sunlight. Imbed in paraffin. By this process the *limits* of the follicle cells are demonstrated.

630. Buds.—Pizon (*Ann. Sc. Nat.*, xix, 1893, p. 5) studies the gemmation of the composite Ascidians either on entire corms, which he first bleaches with peroxide of hydrogen (which acts less brutally than *eau de Javelle*, but the bubbles that arise must be removed with an air-pump), and then stains; or by making sections, after anæsthetising the colonies with cocain of 1 : 1000, fixing in glacial acetic acid or picro-sulphuric or liquid of Flemming, and staining *in toto* with borax carmine or alum carmine, or with a strong solution of methylen blue in alcohol of 90 or 100 per cent. (after Bernard, *ibid.*, ix, 1890, p. 97).

Ritter (*Journ. of Morph.*, xii, 1896, p. 150) recommends for fixing *Perophora* and *Goodsiria* picro-sulphuric acid.

Bryozoa.

631. Statoblasts.—Braem (*Bibl. Zool., Chun and Leuckart*, 6 Heft, 1890, p. 95) fixes statoblasts of *Cristatella* with hot concentrated solution of sublimate for ten minutes, brings them into water and there incises them with a razor, and after half an hour passes them gradually into alcohol. He stains with picro-carmine.

Mollusca.

632. Cephalopoda (Ussow, *Arch. de Biol.*, ii, 1881, p. 582).—Segmenting ova are placed, without removal of the membranes, in 2 per cent. solution of chromic acid for two minutes, and then in distilled water, to which a little acetic acid (one drop to a watch-glassful) has been added, for two minutes. If an incision be now made into the egg-membrane the yolk flows away and the blastoderm remains; if any yolk still cling to it, it may be removed by pouring away the water and adding more.

Watasé (*Journ. of Morphol.*, iv, 1891, p. 249) kills the ova in the macerating mixture of the Hertwigs (§ 543), and as soon as the blastoderm turns white and opaque removes it under dilute glycerin. Treatment with liquid of Perényi is recommended for surface views.

Vialleton (*Ann. Sc. Nat.*, vi, 1887, p. 168) brings ovarian ova of Sepia into a freshly-prepared mixture of picro-sulphuric acid and 2 per cent. solution of bichromate of potash in equal parts, and after one or two minutes incises them in the equator, fixes for an hour and a half in picro-sulphuric acid the halves that contain the formative vitellus, separates this from the nutritive vitellus with a spatula, spreads it out, and hardens it in alcohol of 70 to 90 per cent. He fixes entire ova in liquid of Flemming or osmic acid.

Korschelt (*Festschrift Leuckart*, Leipzig, 1892, p. 348) fixes advanced embryos of Loligo in liquid of Flemming, sublimate, picro-sulphuric acid, or 0·2 per cent. chromic acid. This last is specially good for young embryos if it is washed out with many changes of picric acid.

Faussek (*Mitth. Zool. Stat. Neapel*, xiv, 1900, p. 83) recommends particularly picro-nitric acid. Fix in this, harden in alcohol, bring the ova, still in their albumen, into hæmalum, stain for 24 hours, wash in 1 per cent. alum solution for 24 hours, when the albumen will be found softened so that the ova can easily be extracted.

633. Gastropoda (Henneguy).—Ova of *Helix* may be fixed for from four to six hours in Mayer's picro-nitric acid. The carbonate of lime that encrusts the external membrane is thus dissolved, and the albuminous coat of the egg is coagulated. The egg is opened with needles, the albumen comes away in bits, and the embryo can be removed. Treat with successive alcohols, and imbed in paraffin.

Miss A. Henchman (*Bull. Mus. Comp. Zool., Harvard*, xx, 1890, p. 171) fixes ova of *Limax* with 0·33 per cent. chromic acid, or with liquid of Perényi. It is best to remove only the outer envelope before putting into the chromic acid, the inner membrane being removed after two or three minutes therein. Where Perényi is used the membranes must be removed first, as the albumen will else coagulate in such a way as to prevent the removal of the embryos. For the manner of obtaining the ova, see *previous editions*.

Meisenheimer (*Zeit. wiss. Zool.*, lxii, 1896, p. 417) dissects out the embryos of *Limax* and fixes them with picro-sulphuric acid or concentrated sublimate. Advanced embryos are first got into extension by means of 2 per cent. cocain, or are rapidly killed with hot sublimate.

Schmidt (*Entw. Pulmonaten*, Dorpat, 1891, p. 4) fixes the ova *in toto* with concentrated sublimate, and dissects them out afterwards.

Similarly Kofoid (*Bull. Mus. Harvard Coll.*, xxvii, 1895, p. 35). Or, preferably, the ova are put into salt solution, the shell removed, the albumen removed with a pipette full of salt solution, which dissolves it; the ova are then fixed for one minute in Fol's modification of liquid of Flemming, and brought direct into Orth's picro-lithium-carmine. See also Linville, *ibid.*, 1900, p. 215, who adopts this method of shelling, but prefers fixing in acetic-acid-sublimate, or liquid of Perényi.

Byrnes (*Journ. of Morph.*, xvi, 1899, p. 201) fixes ova until they appear opaque in sublimate (saturated with 5 per cent. acetic acid), puts into water and opens the capsule before removing the albumen, then puts for quarter of an hour into liquid of Flemming.

Holmes (*ibid.*, 1900, p. 371) teases the egg-capsules of *Planorbis* in nitrate of silver of ¾ per cent., exposes to sunlight until the cell-limits come out, rinses with 0·2 per cent. hyposulphite of soda, puts for a few minutes into picric acid, and then through alcohol into balsam.

See also Washburn, *Amer. Nat.*, xxviii, 1894, p. 528 (liquid of Flemming or 0·3 per cent. chromic acid, or 1 per cent. osmic acid, followed by liquid of Merkel).

Conklin (*Journ. of Morph.*, xiii, 1897, p. 7) fixes ova of *Crepidula* for fifteen to thirty minutes in picro-sulphuric acid, and stains with dilute acidified hæmatoxylin of Delafield.

Kostanecki and Wierzejski (*Arch. mik. Anat.*, xlvii, 1896, p. 313) fix the spawn of *Physa fontinalis* either in 1½ to 2 per cent. nitric acid, or in "sublimate and 3 per cent. nitric acid in the proportion of 2 : 1," and bring through successive alcohols. They imbed entire ova in paraffin, but isolated embryos in celloidin.

634. Chiton, see Metcalf, *Stud. Biol. Lab. Johns Hopkins Univ.*, v, 1893, p. 251.

635. Lamellibranchiata.—Stauffacher (*Jena Zeit.*, xxviii, 1893, p. 196) fixes embryos of *Cyclas* in sublimate, stains with hæmalum, and cuts in paraffin.

Lillie (*Journ. of Morph.*, x, 1895, p. 7) fixes ova of *Unio* for ten to twenty minutes in liquid of Perényi, and preserves them in 70 per cent. alcohol, or advanced embryos with liquid of Merkel or sublimate, larvæ with 0·05 to 0·1 per cent. osmic acid, preserving them in glycerin. Glochidia may be cut with the shell in paraffin of 58° melting-point; they may be anæsthetised with chloral hydrate before fixing.

Arthropoda.

636. Fixation of Ova.—In most cases the ova of Arthropods are fixed by heat (§ 11), in a more satisfactory way than by any other means. This may be followed either by alcohol or some watery hardening agent. If it be desired to avoid heating, picro-sulphuric acid may be tried.

637. Removal of Membranes.—This is frequently very difficult, and it may often be advisable not to attempt to remove them, but to soften them with *eau de Javelle* or *eau de Labarraque* (see § 566).

MORGAN (*Amer. Natural.*, xxii, 1888, p. 357) recommends (for the ova of *Periplaneta*) *eau de Labarraque* diluted with five to eight volumes of water, and slightly warmed. Thus used it will soften the chitin membranes sufficiently in thirty to sixty minutes, if employed before fixing. Fixed ova take longer. The fluid must, of course, not be allowed to penetrate into the interior of the ovum.

638. HENKING'S **Methods** (*Zeit. wiss. Mik.*, viii, 1891, p. 156).—HENKING generally kills ova by plunging them into hot water, or by pouring hot water on to them in a watch-glass, and then removing into 70 per cent. alcohol.

He thinks that *eau de Javelle* for softening membranes is best avoided. They should either be dissected away or left *in situ*, and cut with the rest of the egg, according to the nature of the case. The great obstacle to section-cutting is the brittleness of the yoke. This difficulty may be overcome as follows: After fixing and treating with alcohol, prick the chorion and stain with borax-carmine. Put the stained ova for twelve hours into a mixture containing 20 c.c. of 70 per cent. alcohol, one drop of concentrated hydrochloric acid, and a knife pointful of pepsin (it is not necessary that all the pepsin should be dissolved). The ova may then be treated with alcohol, oil of bergamot, and paraffin, and (with some exceptions, amongst which is *Bombyx mori*) will be found to cut without crumbling.

639. Diptera (HENKING, *Zeit. wiss. Zool.*, xlvi, 1888, p. 289).—Ova still contained within the fly may be fixed by plunging the animal for some time into boiling water, then dissecting out and bringing them into 70 per cent. alcohol. Laid eggs may have boiling water poured over them, or be put into solution of Flemming in a test-tube which is plunged into boiling water until the eggs begin to darken (about a minute). Cold solution of Flemming easily causes a certain vacuolisation of the contents of the ova. Open the ova at the larger end, stain with borax-carmine for fifteen to thirty hours, and cut in paraffin.

BRUEL (*Zool. Jahrb., Abth. Morph.*, x, 1897, p. 569) fixes larvæ and pupæ in absolute alcohol heated to 70° to 75° C., and containing "a little" sublimate. See also VAN REES, *ibid.*, iii, 1888, p. 10.

BENGTSSON (*Handl. Fysiogr. Sællsk Lund.*, viii, 1897) finds hot alcoholic solution of sublimate (Frenzel's, § 74) the best fixative for larvæ of *Phalacrocera*. He could not succeed in softening the chitin with *eau de Javelle.*

Similarly BERLESE (*Riv. di Patol. Veget.*, viii, 1899) for larvæ and nymphs of Muscidæ, leaving them in the sublimate for two or three hours at a temperature of 45° C.; then iodine alcohol, benzine, and paraffin.

640. Lepidoptera (BOBRETZKY, *Zeit. wiss. Zool.*, 1879, p. 198).—Ova are slightly warmed in water and put for sixteen to twenty hours in 0·5 per cent. chromic acid. The membranes can then be removed.

641. Blattida (PATTEN, *Quart. Journ. Mic. Sci.*, 1884, p. 549).—The ova or larvæ are placed in cold water, which is gradually raised to 80° C. You leave off heating as soon as the ova have become hard and white. Pass very gradually through successive alcohols, beginning with 20 per cent.

WHEELER (*Journ. of Morph.*, iii, 1889, p. 292) dissects out ovarian ova in salt solution and fixes in liquid of Perényi (fifteen minutes), then treats with alcohol, and stains with borax-carmine. Laid eggs may be killed by Patten's method. After heating, the two lips of the crista of the capsule may be separated with fine forceps and pieces of the walls torn away, and the eggs pushed out of the compartments formed by their choria and hardened as desired. Good results are also obtained by heating to 80° C. for ten minutes in liquid of Kleinenberg, and preserving in 70 per cent. alcohol. This causes the envelopes to dilate and stand off from the surface of the egg, so that they can easily be dissected away.

Heymons (*Zeit. wiss. Zool.*, liii, 1892, p. 434), for young embryos, incises the cocoon at the end by which it adheres in the body of the mother, brings it for two minutes into water heated to 90° C., and opens in Flemming, in which the embryo is dissected out.

Morgan (*Amer. Natural.*, xxii, 1888, p. 357) puts ova of *Periplaneta* for thirty minutes or an hour into eau de Javelle diluted with 4 to 8 vols. of water and slightly warmed, which softens the capsules.

642. Phalangida.—The ova of *Plalangium opilio* possess a chorion covered with yellow corpuscles that render them opaque. Balbiani puts them into water with a few drops of caustic potash, and raises to boiling point. The ova are then laid on filter paper, and the chorion removed by rubbing with a camel's hair brush, the vitelline membrane remaining intact, so that the embryo can be studied through it.

Henking's method (*Zeit. wiss. Zool.*, xlv, 1886, p. 86).—Fix with boiling water or Flemming. Preserve the ova in 90 per cent. alcohol. To open the chorion, bring them back into 70 per cent. alcohol, which causes them to swell up so that the chorion can easily be pierced with needles, and the ovum turned out.

643. Araneida.—Kishinouye (*Journ. Coll. Sci. Imp. Univ. Japan*, iv, 1891, p. 55; *Zeit. wiss. Mik.*, ix, 1892, p. 215) fixes in water warmed to 70° or 80° C., puts into 70 per cent. alcohol, and after twenty-four hours therein pierces the membranes and passes through stronger alcohol.

See also Locy, *Bull. Mus. Comp. Zool., Harvard*, xii, 3, 1886. Fix by hot water. The liquid of Perényi may also be used; it has the advantage of not making the yolk so granular.

644. Decapoda.—Reichenbach (*Abh. Senckenberg. Ges. Frankfurt*, xiv, 1886, p. 2) fixes ova of *Astacus* in water gradually warmed to 60° or 70° C. (if the chorion should burst, that is no evil), hardens for twenty-four hours in 1 to 2 per cent. bichromate of potash or 0·5 per cent. chromic acid, washes out for the same time in running water, and brings into alcohol. Remove the chorion, and remove the embryo from the yolk by means of a sharp knife.

HERRICK (*Bull. U. S. Fish Comm.*, xv, 1896, p. 226) kills the ova in hot water, shells, and fixes in picro-sulphuric acid.

For *Homarus*, see WAITE, *Bull. Mus. Comp. Zool.*, xxxv, 1899, p. 155.

645. Amphipoda.—DELLA VALLE (*Fauna u. Flora Golf. Neapel*, xx, Monog., 1893, p. 170) puts ova of *Orchestia* by means of a pipette into boiling, cold-saturated sublimate solution, removes them instantly into sea water, and thence into weak alcohol. If the chorion does not burst of itself it must be pricked with a needle.

Vermes.

646. Rotatoria.—JENNINGS (*Bull. Mus. Harvard Coll.*, xxx, 1896, p. 101) finds the best fixative for pregnant females is the strong liquid of Flemming, but the ova must then be bleached with chlorate of potash (§ 587).

647. Turbellaria.—GARDINER (*Journ. of Morph.*, xi, 1895, p. 158) finds the best fixative for ova of *Polychoerus* is a mixture of equal parts of absolute alcohol and glacial acetic acid.

Method of IIJIMA (*Zeit. wiss. Zool.*, xl, 1884, p. 359).—The capsule containing the ova (of fresh-water Planaria) is opened with needles on a slide, in a drop of 2 per cent. nitric acid. The ova are extracted and covered (the cover being supported by paper or by wax feet). After half an hour they are treated with successive alcohols under the cover, and finally mounted in glycerin. For sections, the whole of the contents of a capsule is hardened in the mass in 1 per cent. chromic acid and cut together.

VANDER STRICHT (*Arch. Biol.*, xv, 1898, p. 370) finds that ova of *Thysanozoon* will only cut well when they have been not more than two minutes in absolute alcohol followed by chloroform and paraffin as used by Carnoy and Lebrun, § 614.

See also, for Polyclads, FRANCOTTE, *Arch. Zool. Expér.*, vi, 1898, p. 196.

648. Cestoda (v. Beneden, *Arch. Biol.*, ii, 1881, p. 187).—Ova of *Tænia* in which a chitinous membrane has formed around the embryo are impervious to reagents. They may be put on a slide with a drop of some liquid and covered. Then, by withdrawing the liquid by means of blotting-paper, the cover may be made to gradually press on them so as to burst the membranes, and the embryo may then be treated with the usual reagents.

649. Trematoda.—Coe (*Zool. Jahrb. Abth. Morph.*, ix, 1896, pp. 563, 566) fixes the Miracidia of Distomum for general purposes with the usual fixatives; but for the special study of the excretory system he kills them with osmic acid, rinses with distilled water, and puts them for a couple of days into ¼ per cent. solution of silver nitrate.

Egg-capsules may be softened with 5 per cent. caustic potash and then burst open (Heckert, *Bibl. Zool.*, iv, 1889).

650. Nematoda.—The ova of *Ascaris megalocephala*, a classical object of study, are one of the most impervious things in the animal kingdom. Years ago Fol related to me that he had had ova segmenting right through absolute alcohol into balsam. Bataillon (*Arch. Entwickelungsmech.*, 1901, p. 149) has had ova showing living embryos after having been for six months in liquid of Flemming, and found them to remain alive for months after drying for 24 hours at 35° C., and mounting in balsam, and for weeks in acids or alkalies. It is evident that only extremely penetrating and powerful fixatives can be of any use here.

Doubtless the best fixative yet made known for ova furnished with their capsules will be found to be that of Gilson (§ 91); see Carnoy and Lebrun, *La Cellule*, xiii, 1897, p. 68. After fixation the ova are carefully brought into 80 per cent. alcohol, in which they are preserved. Imbedding should be carefully done as recommended for the ova of Amphibia (§ 614), but they ought not to remain in the pure paraffin for more than a minute to a minute and a half. But these authors prefer the celloidin method. At least six weeks' soaking in the different strengths of celloidin will be necessary to ensure penetration. They stain with iron hæmatoxylin.

ZUR STRASSEN (*Arch. Entwickelungsmech.*, iii, 1896, p. 29) fixes for twenty-four hours in a mixture of 4 parts 96 per cent. alcohol and 1 part acetic acid, brings into pure alcohol, stains with hydrochloric acid carmine, and brings gradually into glycerin.

Similarly ZOJA (*Arch. mik. Anat.*, xlvii, 1896, p. 218) and ERLANGER (*ibid.*, xlix, 1897, p. 309). Zoja stained with Bismarck brown and examined in dilute glycerin; Erlanger made paraffin sections and stained with iron hæmatoxylin.

KOSTANECKI and SIEDLECKI (*ibid.*, xlviii, 1896, p. 184) employed concentrated sublimate solution, or 3 per cent. nitric acid, or mixtures of these two, for ovarian ova.

VAN BENEDEN and NEYT (*Bull. Acad. Belg.*, 1887, p. 214) took equal parts of alcohol and acetic acid.

BOVERI (*Jena Zeit.*, xxi, 1887, p. 423) fixes in his picro-acetic acid, § 100, after which treatment I should say you may easily see anything you like to imagine in the preparations.

Echinodermata, Cœlenterata, and Porifera.

See the chapter on "Zoological Methods."

CHAPTER XXVI.

CYTOLOGICAL METHODS.

651. Study of Living Cells.—In the young larvæ of Amphibia, both Anura and Urodela, the gills and caudal "fin," and sometimes other regions, may be conveniently studied in the living state.

The larvæ may be fixed in a suitable cell, or wrapped in moist blotting-paper, or may be curarised; or the tail may be excised. (It is preferable to cut through the larva close in *front* of the hind limbs.)

In the *living animal* the epithelial cells and nuclei (in the state of repose) are so transparent as to be hardly visible in the natural state. They may, however, be brought out by curarising the larva; or, still better, by placing the curarised larva for half an hour in 1 per cent. chloride of sodium solution. Normal larvæ may be used for the study of the active state of the nucleus, but much time is saved by using curare.

Curare.—Dissolve 1 part of curare in 100 parts water, and add 100 parts of glycerin. Of this mixture add from 5 to 10 drops (according to the size of the larva), or even more for large larvæ, to a watch-glassful of water. From half to one hour of immersion is necessary for curarisation. The larvæ need not be left in the solution until they become quite motionless; as soon as their movements have become slow they may be taken out and placed on a slide, wrapped in blotting-paper. If they be replaced in water they return to the normal state in eight or ten hours, and may be re-curarised several times.

Etherisation.—Three per cent. alcohol or 3 per cent. ether may be used in a similar way. These reagents cause no obstruction to the processes of cell-division, and are useful, but their action as anæsthetics is inconstant.

Tobacco.—Larvæ of Amphibia become immobilised in a few minutes in an infusion of tobacco, and recover after a few hours if put back into water.

Indifferent Media.—One per cent. salt solution, iodised serum, syrup, cold water (+ 1° C.), and warm water (35°—40° C.). The tail may be excised from the living animal and studied for a long time in these media (PEREMESCHKO, *Arch. mik. Anat.*, xvi, 1879, p. 437).

Small and transparent aquatic organisms, such as larvæ of Diptera, small specimens of *Clepsine* and *Nephelis*, etc., may be studied alive in a reversible compressorium. The vegetable kingdom also affords some good objects, for which see the botanical treatises.

For the processes of staining living cells see § 221.

652. Study of Fresh and Lightly Fixed Cells.—So-called "indifferent" liquids must not be believed to be without action on nuclei. Iodised serum, salt solution, serum, aqueous humour, lymph, better deserve the name of weak hardening agents. Between these and such energetic hardening agents as Flemming's mixture come such light fixing agents as picric acid or very dilute acetic acid. These it is whose employment is indicated for the study of fresh isolated cells.

A typical example of this kind of work is as follows: Tease out a piece of living tissue in a drop of acidulated solution of methyl green (0·75 per cent. of acetic acid). This is a delicate fixing agent, killing cells instantly without change of form. Complete the fixation by exposing the preparation for a quarter of an hour to vapour of osmium, and add a drop of solution of Ripart and Petit and a cover.

Or you may fix the preparation, after teasing, with vapour of osmium for half a minute to two minutes, then add a drop of methyl green, and after five minutes wash out with 1 per. cent acetic acid, and add solution of Ripart and Petit and cover.

Or you may kill and fix the cells by teasing in solution of Ripart and Petit (to which you may add a trace of osmic acid if you like), and afterwards stain with methyl green.

I have found Pictet's chloride of manganese (§ 413) useful as an examination medium. A little solution of dahlia may be added to it.

Henking (*Zeit. wiss. Mik.*, viii, 1891, p. 156) recommends a liquid composed of

Water	80 c.c.
Glycerin	16 ,,
Formic acid	3 ,,
Osmic acid of 1 per cent.	1 ,,
Dahlia	0·04 grm.

Other fixing agents, such as picric acid or weak sublimate solution, may of course be used, and in some cases doubtless should be preferred. Other stains, too, such as Bismarck brown, may be used as occasion dictates; and of course other examination media than solution of Ripart may be employed. But, for general purposes, the methyl-green-osmium-and-Ripart's-medium method gives such good results, and is so very convenient, that it may be called a classical method for the study of fresh cells.

653. Some Microchemical Reactions.—*Methyl green* is a test for chromatin, in so far as it colours nothing but the chromatin *in the nucleus, see* § 288. It is, however, not a perfect test, for the intensity of the coloration it produces varies greatly in different nuclei, and may in certain nuclei be extremely weak, or (apparently) even altogether wanting. In these cases other tests must be applied in order to establish with certainty the presence or absence of that element. The following suggestions are taken from Carnoy, who is, I believe, the only writer—on the zoological side, at all events—who has insisted on the necessity of applying microchemical methods in a systematic manner to the study of cells.

Chromatin is distinguished from albuminoids by not being soluble, as these are, in water and in weak mineral acids, such as 0·1 per cent. hydrochloric acid. It is easily soluble in concentrated mineral acids, in alkalies, even when very dilute, and in some alkaline salts, such as carbonate of potash and biphosphate of soda. In the presence of 10 per cent. solution of sodium chloride it swells up into a gelatinous mass, or even, as frequently happens, dissolves entirely (*Biol. Cell.*, pp. 208–9). It is only partially digestible (when *in situ* in the nucleus) in the usual laboratory digestion fluids.

The solvents of chromatin that are the most useful in practice are 1 per cent. caustic potash, fuming hydrochloric acid, or cyanide of potassium, or carbonate of potash. These last generally give better results than dilute alkalies. They may be employed in solutions of 40 to 50 per cent. strength. If it be desired to remove all the chromatin from a nucleus the reaction must be prolonged, sometimes to as much as two or three days, especially if the operation be conducted on a slide and under a cover-glass, which is the safer plan.

It must be remembered that these operations must be performed on *fresh* cells, for hardening agents bring about very considerable modifications in the nature of chromatin, rendering it almost insoluble in ammonia, potash, or sodic phosphate, etc. Hydrochloric acid, however, still swells and dissolves it, though with difficulty.

Partial digestion may render service in the study of the chromatic elements of nuclei. Chromatin resists the action of digestive fluids much longer than the albumins do; so that a moderate digestion serves to free the chromosomes from any caryoplasmic granulations that may obscure them, whilst at the same time it clears up the cytoplasm.

Concerning the microchemistry of the cell see further *fourth edition*; also CARNOY & LEBRUN, *La Cellule*, xii, 2, 1897, p. 194; ZIMMERMANN, *Die Morphologie u. Physiologie des Pflanzlichen Zellkernes*, Jena, 1896 (treats also of the animal cell); HAECKER, *Praxis u. Theorie der Zellen- und Befruchtungslehre*, Jena, 1899.

654. Cytological Fixing Agents.—It does not follow that a fixing agent that is good for one element of a cell is also good for all others. That which is good for cytoplasm is not necessarily good for the nucleus, and *vice versâ*.

As regards the nucleus, it is a rule that admits of no exception that all fixatives must be *acid*; for if not they will not satisfactorily preserve either chromatin or nucleoli.

For instance, bichromate of potash, if not rendered acid, should be banished from the study of nuclei, because it causes chromatin and nucleoli to swell, so that clear images of them are not obtained. (I do not myself think that, as regards chromosomes at all events, the images given by bichromate are so *unnatural* as they are held to be by Flemming and the majority of authors. Chromosomes during life are always in a state that may be fittingly described as *swollen* by comparison with their state after fixation by acids. During life, in the equatorial and polar stages of division, they are mostly compacted into plates or pectiniform figures in which the separate elements are not clearly discernible, and which are more like the images given by fixation with bichromate than those which are given by fixation with acids. The acids contract them somewhat, and so give them sharper outlines, and thus render them individually distinguishable. The resulting image thus becomes clearer, but I do not admit that it is more *lifelike*.)

The fixatives chiefly employed for nuclei are liquid of Flemming and liquid of Hermann, § 50. For most purposes I think they are as good as anything that has hitherto been imagined. There is a slight difference between them. Liquid of Hermann, owing to the platinum chloride, causes chromatin to shrink more than liquid of Flemming does, and for this reason is supposed to give clearer images of chromosomes, especially of their splitting. I find that it generally makes them shrink *too much*, and that it is not at all good for spindles.

Alongside of these two reagents I would place Bouin's picro-formol, § 117, which gives a highly faithful preservation and a more penetrating and equable fixation.

But it is a mistake to suppose that equally good images cannot be obtained by means of other reagents. Some of the finest chromosomes I have seen have been fixed with Lindsay Johnson's mixture (§ 49), and liquid of Tellyesniczky has given me others nearly if not quite as good. Mixture of Gilson, § 91, also gives very fair nuclei indeed, and is highly useful where very great penetration is required.

In these remarks I have left the caryoplasm, or whatever else there may be in a nucleus, besides the chromatin and nucleoli, out of account, as next to nothing is known concerning it.

As regards the cytoplasm. Cytoplasm is made up of two elements, a fibrillar network—the spongioplasm, reticulum, or mitome; and a more or less granular liquid that bathes it—the hyaloplasm or enchylema. It does not follow that a reagent that will fix one of these will also fix the other. Nor does it follow that if both are fixed you have of necessity a perfect fixation, for that depends on the object in view.

If you fix both, you will have a *full* fixation; but in that case the granules of the hyaloplasm (be they vital, or be they only "precipitation forms," see § 27), and the secretions or other inclosures that may be present in it, may so mask the fibrils of the spongioplasm as to interfere with the observation of it. So that if the latter is the principal object of study, a *thin* fixation, one in which the spongioplasm is entirely preserved, but the hyaloplasm only partly, may be the better.

The *spongioplasm* is the easier to fix of the two, and the majority of acid fixatives will preserve it more or less; for instance, the osmic acid, chromic, or picric mixtures, or corrosive sublimate. The best images I have obtained are those given by liquid of Flemming or Hermann in cells in which the action of the reagent has been moderate, *i.e.* insufficient to thoroughly fix the hyaloplasm at the same time. As to the latter reagent, see § 50. Nearly, if not quite, as good, is Bouin's picro-formol, § 117, which has the great advantage of being very favourable for plasma-staining. I have also had good results with vom Rath's picro-osmic and picro-platinosmic mixtures, and with acid sublimate.

Hyaloplasm is not nearly so easy to fix, and there are only two reagents in common use that readily give a really full fixation of it; these are *osmic acid* and *bichromate of potash*.

Osmic acid acts as a fixative of hyaloplasm in liquid of Flemming or Hermann, but only gives a full fixation in the outer layers of the material; and in these it easily happens that many or most of the cells are ruined by over-fixation (see §§ 28, 39).

This defect may be to a certain degree corrected by taking the osmic acid weaker than is usual. Thus by successively reducing the proportion of this ingredient in liquid of Hermann,* I have found that it can be brought down to

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* Niessing (*Arch. mik. Anat.*, xlvi, 1895, p. 147) has the following two modifications of Hermann's mixture:

(1)	Platinum chloride, 10 per cent. solution . .	25
	Osmic acid, 2 per cent.	20
	Glacial acetic acid	5
	Distilled water	50

(2) The same with saturated aqueous solution of corrosive sublimate instead of the water.

They are both of them, in my opinion, as ill-imagined as possible. They contain some three times as much platinum chloride as Hermann's, *one eighth* of the prescribed amount without loss of the distinctive characters of the fixation. But it cannot be entirely omitted without the character of the fixation changing altogether.

The defect of want of penetration seems to be incurable (see §§ 28, 39, and 47). Substitution of more highly penetrating reagents, such as picric acid, for the chromic acid or platinum chloride, does not help in the least; you only get the osmic fixation outside, no whit deeper than before, and a picro-acetic fixation, instead of a chromo- or platino-acetic one, in the deeper layers, that is all. For instance, vom Rath's picro-platinosmic mixture, § 108, may often give better results in some respects than liquid of Hermann; but that is not on account of the addition of the picric acid, it is rather on account of the platinum chloride being taken weaker. The *osmic* fixation is not in the least modified by the picric acid in it.

In view, then, of these defects of osmic mixtures, it may often be advisable, where hyaloplasm, or its enclosures, is the chief object of study, to have recourse to *bichromate of potash*. The formula that has given me the finest fixations is that of LINDSAY JOHNSON, § 49, but it has the drawback that there is risk of osmication in the outer layers.

In this respect liquid of Tellyesniczky, § 56, is to be preferred.

Corrosive sublimate gives a fairly full fixation; but I believe it frequently produces serious artefacts, HEIDENHAIN's "Lanthanin" being one of them. Heidenhain's solution, § 70, containing as it does some 11 per cent. of sublimate, without the addition of any acid to neutralise its shrinking action, seems to me to be an inadmissibly coarse reagent. I have, however, obtained with liquid of Gilson-Carnoy-Lebrun, § 91, some most excellent fixations of cytoplasm. The aqueous solutions of sublimate are frequently used in preference to liquid of Flemming on account of the facilities they afford for the employment of certain stains; but to that end I prefer BOUIN's picro-formol, § 117.

and Hermann's contains already quite as much as it can bear, and, I think, much more than is advisable: see the proportions in the mixtures §§ 49 and 53. RABL (*Anat. Anz.*, iv, 1889, p. 21) employed it of from $\frac{1}{10}$ to $\frac{1}{8}$ per cent. strength, which seems to me much nearer the mark.

ALTMANN's fixatives for nuclei see *last edition*, or *Arch. Anat. Entw.*, 1892, p. 223, and his *Elementarorganismen*, 1890. His mixture for his granula see § 48. See also THÉOHARI (*Journ. de l'Anat.*, xxxvi, 1900, p. 216).

655. Chromatin Stains.—For fresh tissues see § 652.

For sections of hardened tissues, stains should be chosen amongst those that give a very intense as well as a very sharp coloration. Some years ago safranin and gentian violet, § § 298, 299, were the most used. At the present time their place has been taken by the iron hæmatoxylin of BENDA or HEIDENHAIN.

See also Thionin, § 300, Keruschwarz, § 386, and Iron Carmine, § 236.

For some remarks of BATAILLON and KOEHLER on the stain of borax-methylen-blue see *Comptes Rendus*, cxvii, 1893, p. 521, or *Journ. Roy. Mic. Soc.*, 1894, p. 41.

656. Plasma Stains.—I have been unable to discover a single thoroughly satisfactory one. Almost all of them colour too readily the enchylema or hyaloplasm at the same time as the plasmatic reticulum. And, on the other hand, there are many important elements of cells which cannot be got to stain sufficiently.

I consider Säurefuchsin, § 303, the most generally recommendable. It works particularly well after iron hæmatoxylin. See also orange G., § 304.

Flemming's Orange Method, § 310, has been much used. I do not recommend it, as it is very capricious and unreliable. Benda's *Safranin and Lightgrün or Säureviolett*, § 323, gives sometimes splendid results, but is capricious.

Ehrlich-Biondi mixture is a celebrated plasma stain. See § 306.

The *Osmic Acid and Pyrogallol Process*, § 383, gives a very fair and frequently useful plasma stain ; but I do not consider it to be a method of quite the first class.

The *Iron-Hæmatein Lakes* of Benda and M. Heidenhain give good plasma stains, according to the degree of extraction, and would be inferior to none were it not that they stain in the same tone as the chromatin. See also Ehrlich's *tri-acid*, § 307, and his *acidophilous mixture*, § 320 ; also

Wasserblau, § 335, *gold chloride*, Apáthy's process, § 380, and *Kernschwarz*, § 386.

657. Centrosomes.—The formations known as "centrosomes," "central corpuscles," "centrioles," "polar corpuscles," *can* be stained by some "acid" anilin dyes, better by a "neutral" dye (*e. g.* Flemming's orange method, § 310). But *by far the best* stain is iron-hæmatoxylin. See § 260.

It is said by Heidenhain that the stain is obtained in a sharper form by combining the hæmatein stain with a foregoing stain with *Bordeaux R.* He directs (*Arch. mik. Anat.*, xlii, 1894, p. 665) that the sections (sublimate sections were used by him) are to be stained for twenty-four hours or more in "a weak" solution of Bordeaux, until they have attained such an intensity of colour as that "they would just be fit for microscopic examination with high powers" (*l. c.*, p. 440, note), and that they be then brought into the ferric alum. After mordanting and staining, the hæmatein is to be extracted in the iron alum until the chromatin has become entirely or almost entirely colourless. Instead of Bordeaux, "anilin blue" may be used in the same way. I think the advantages of the process have been over-rated.

The images of these objects given by iron-hæmatoxylin require to be interpretated with special care. For they sometimes exhibit the phenomenon termed by Fischer (*Fixirung, Färbung und Bau des Protoplasmas*, 1899, p. 31, *et passim*) "Spiegelfaerbung," that is—a *bull's eye effect.* Owing to the rapidity of the decoloration in the differentiating liquid, globular or even elongated objects, such as chromosomes, do not always lose their stain simultaneously and gradually throughout their whole depth, but yield it up suddenly and entirely in their outer layers, whilst retaining it in its full strength in their deeper layers. The still-stained parts thus remain separated from the decoloured parts by a sharply-defined limit, with no penumbral zone of transition; so that a spherical granule in this state will show a central point deeply stained—the bull's eye—and around it a perfectly colourless area—the white of the target. And when the object is in balsam it is frequently quite impossible to distinguish the outer limit of this colourless area, so that the whole object appears to have only the dimensions of the stained area. It seems that certain erroneous observations that have been published have been due to this deception.

Hermann (*Arch. mik. Anat.*, xxxvii, 4, 1891, p. 583) recommends a modification of the hæmatoxylin impregnation method of **Pal**, for which see *fourth edition*; also his paper, "Methoden zum Studium des Archoplasmas" in *Ergebnisse der Anatomie*, Band ii, 1892 (1893), p. 23.

For **Heidenhain's** *Vanadium hæmatoxylin*, see § 284.

Benda (*Verh. Phys. Ges. Berlin*, November, 1900, Nr. 1–2; *Verh. Anat. Ges.*, xv, 1901, p. 167) gives the following as succedanea of the iron hæmatoxylin method: The material is to be fixed in alcohol of about 93 per cent. for two days, then treated for twenty-four hours with nitric acid diluted with 10 vols. of water. Then bichromate of potash of 2 per cent., twenty-four hours; chromic acid of 1 per cent., forty-eight hours; water, twenty-four hours; alcohol; paraffin (or sections by freezing). The sections are stained either by iron hæmatoxylin, § 259 (twenty-four hours in the mordant and in the stain, with differentiation in Weigert's borax-ferricyanide), or by either of the two following methods:

(*a*) The sections are oxidised for five minutes in 0·5 per cent. solution of permanganate of potash, reduced in **Pal's** oxalic mixture till they become white (about three minutes), dried with blotting-paper, flooded with **Weigert's** methyl-violet-oxalic mixture, or with the crystal-violet solution, § 337, dried, rinsed with solution of **Lugol**, rinsed, dried again with blotting-paper, differentiated with a mixture of equal parts of xylol and anilin oil, dried, rinsed with xylol, balsam.

(*b*) Sections mordanted twenty-four hours in iron alum of 4 per cent. or *liquor ferri*, § 259, diluted with 2 vols of water, rinsed, stained twenty-four hours in the sol. of sulphalizarinate of soda, § 344, rinsed, mopped with blotting-paper, warmed in 0·1 per cent. sol. of toluidin blue till vapour is given off, stained fifteen minutes more in the same solution whilst cooling, dipped in acetic acid of 1 per cent., dried with blotting-paper, dipped in alcohol, differentiated about ten minutes in beech-wood creosote, dried with blotting-paper, rinsed many times with xylol, then balsam.

658. Cell Granules.—For the study of the conspicuous "granules," undoubtedly metabolic products, occurring in certain gland-cells and blood- and lymph-corpuscles, and in certain elements belonging to the group of connective tissues, see the sections on "Connective Tissues." The most generally employed stains are the mixtures of **Ehrlich**.

Intra vitam staining is useful here (see § 221). See also **Arnold**, *Anat. Anz.*, xxi, 1902, p. 417.

Benda (*Verh. phys. Ges. Berlin*, 1899-1900, No. 1-4, and *Verh. Anat. Ges.*, xv, 1901, p. 172) gives the following method for demonstrating *secretion-granules* and distinguishing them from other granules: Harden for 24 hours in 10 per cent. formalin, then for one day in 0·25 per cent.

chromic acid, one in 0·33 per cent. and 2 to 3 in 0·5 per cent., wash one day in water, dehydrate and make paraffin sections. Then stain with one of Ehrlich's mixtures, according as the granulations are basophilous, acidophilous, or neutrophilous. The methylen-blue and eosin process of Michaelis is recommended.

For BENDA's latest method for the study of the granulations named by him *Mitochondria*, see § 344.

For PRENANT's *Ergastoplasm* see especially GARNIER, *Bibliogr. Anat.*, Nov. 6th, 1897, p. 278, and *Journ. de l'Anat.*, xxxvi, 1900, p. 22 (fixation in liquid of Flemming, or picro-formol of Bouin, staining by Flemming's orange method, or by iron hæmatoxylin, or by toluidin blue, after mordanting for five or six minutes with diluted tincture of iodine).

For ALTMANN's "Bioblasts" see § 48 and *early editions*, also the critique of FISCHER, in his *Fixirung, Färbung, und Bau des Protoplasmus*, pp. 108, 295.

659. Nucleoli are "acidophilous" in so far as, in fixed material, they select the acid dye or dyes from mixtures such as the Ehrlich-Biondi stain. With this they stain mostly red, sometimes orange. With fresh material they do not stain at all with acid methyl green (distinction from chromatin).

But in fixed material treated with basic dyes (safranin, gentian, etc.) by the regressive method they stain more energetically than resting chromatin, and at least as much so as chromatin in the kinetic state. With iron-hæmatoxylin they stain sometimes full black, sometimes grey with a black shell.

They can frequently be well demonstrated in unstained preparations examined in water, being brought out by their superior refractivity; and are sometimes visible in the living cell.

One of the best ways of demonstrating them is to fix with strong liquid of Flemming, and stain with safranin, followed by differentiation with acid alcohol (§ 298).

See also REDDINGIUS, *Virchow's Arch.*, clxii, 1900, p. 206. For nucleoli of ova, LIST, *Mitth. Zool. Stat. Neapel*, xii, 1896, p. 480; of nerve-cells, RUZICKA, *Zeit. wiss. Mik.*, xiv, 1898, p. 453, and LEVI, *Riv. Pat. Nerv. Ment. Firenze*, iii, 1898, p. 289,

CHAPTER XXVII.

TEGUMENTARY ORGANS.

660. Epithelium.—Both for surface views and for sections good results are obtained by the *nitrate of silver* method, the *methylen blue* method, the *perchloride of iron and pyrogallol* method of the Hoggans, § 384, the *osmic acid and pyrogallol* process, § 383, and by *iron-hæmatoxylin.*

For the purpose of separating the epidermis from the corium, LOEWY (*Arch. mik. Anat.*, xxxvii, 1891, p. 159) recommends macerating for twenty-four to forty-eight hours, at a temperature of about 40° C., in 6 per cent. pyroligneous acid. Acetic acid of $\frac{1}{3}$ per cent. (PHILIPPSON) is also good. MINOT (*Amer. Nat.*, xx, 1886, p. 575) macerates embryos for several days in 0·6 per cent. salt solution, MITROPHANOW (*Zeit. wiss. Mik.*, v, 1888, p. 573) for quarter of an hour in 3 per cent. nitric acid, then 1 hour in one third alcohol, and, if need be, 24 in stronger alcohol.

For *ciliated epithelium* see the methods of Engelmann under "Mollusca."

661. Intercellular Bridges (and Canals).—On this subject (which includes the so-called "Prickle-cells") see the important memoirs of IDE, in *La Cellule,* iv, 1888, p. 409, and v, 1889, p. 321; also KOLOSSOW, *Arch. mik. Anat.*, lii, 1898, p. 1. KOLOSSOW used an osmic-acid-tannin stain, § 383.

See also FLEMMING, *Anat. Hefte,* 1 Abth., vi, 1895, p. 1.

Besides maceration, impregnation may be useful; MITROPHANOW (*Arch. Anat. Phys., Phys. Abth.*, 1884, p. 191) has used gold chloride.

UNNA (*Monatschr. prakt. Dermat.*, xxxvii, 1903, p. 1) has described a highly complicated process with Wasserblau and orcein, see *Zeit. wiss. Mik.*, xxi, 1904, p. 68.

662. Plasma-fibrils of Epithelium.—KROMAYER'S process (*Arch. mik. Anat.*, xxxix, 1892, p. 141) is as follows: Sections are stained for five minutes in a mixture of equal volumes of anilin water (§ 298) and concentrated aqueous solution of methyl violet 6 B. They are well washed in water and treated with solution of iodine in iodide of potassium until they become blue-black (one to thirty seconds). They are again washed with water, dried with blotting-paper, and treated with a mixture of 1 vol. of anilin to 2 vols. of xylol until sufficiently differentiated, when they are brought into pure xylol. Very thin sections will require more xylol in proportion to the anilin, viz. 1 : 3 or 1 : 4 ; thicker ones may require more anilin, viz. 3 : 5 or 3 : 3. Gentian or Krystall-violett will do instead of methyl violet, but not quite so well. For some variations see *Dermatol. Zeit.*, iv, 1897, p. 335 ; *Zeit. wiss. Mik.*, xiv, 3, 1897, p. 396 ; further, EHRMANN and JADASSOHN, *Arch. Dermatol. u. Syphilis*, 1892, 1, p. 303 ; *Zeit. wiss. Mik.*, ix, 1893, p. 356 ; HERXHEIMER u. MÜLLER, *Arch. Dermatol.*, xxxvi, 1896, p. 93 ; *Zeit. wiss. Mik.*, xiv, 2, 1897, p. 216 (they used Weigert's Neuroglia stain) ; SCHÜTZ, *ibid.*, pp. 111, 218 ; and HERXHEIMER, *Arch. mik. Anat.*, liii, 1899, p. 510.

For the same object UNNA (*Monatssch. prakt. Dermatol.*, xix, 1894, p. 1 and p. 277, *et seq.* ; *Zeit. wiss. Mik.*, xii, 1, 1895, pp. 61, 63) has given a whole series of minutely detailed methods, from which the following are some extracts.

(1) WASSERBLAU-ORCEIN.—Stain sections for ten minutes in a neutral aqueous 1 per cent. solution of Wasserblau, rinse with water and stain for five or ten minutes in a neutral alcoholic 1 per cent. solution of Grübler's orcein. Dehydrate, clear, and mount in balsam. This method may be varied as follows :

(*a*) Ten minutes in the Wasserblau and thirty minutes or more in the orcein.

(*b*) Take for the second stain an *acid* solution of orcein.

(*c*) Stain for only one minute in the Wasserblau, but for thirty or more in the neutral orcein.

(2) Stain for half an hour or more in a strong solution of hæmalum, rinse with water, stain for half a minute in a saturated aqueous solution of picric acid, and dehydrate for thirty seconds in alcohol containing 0·5 per cent. of picric acid.

(3) Hæmalum for two hours, neutral orcein as above for ten to twenty minutes.

More recently UNNA advocates the process mentioned last §.

See also RANVIER, *Arch. Anat. Micr.*, iii, 1899, p. 1.

663. Keratohyalin.—The keratohyalin granules of the cells of the *stratum granulosum* are soluble in mineral acids, and can be digested in pepsin. They can be stained with picro-carmine, alum hæmatoxylin, van Gieson's mixture, or Unna's Wasserblau-orcein, last §. FICK (*Centralb. allg. Path.*, xiii, 1902, p. 987; *Zeit. wiss. Mik.*, xx, 1903, p. 222) stains sections of alcohol material for three to four minutes in concentrated aqueous solution of *Kresylechtviolett*, differentiates in alcohol, clears in xylol, and mounts in balsam, which gives a reddish metachromatic stain of the granules.

See also UNNA, *Monatsschr. prakt. Dermat.*, xx, 1895, p. 69 (*Zeit. wiss. Mik.*, xiii, 1897, p. 337), and the article "Haut" in the *Encycl. mik. Technik.*

664. Eleidin.—To demonstrate the *stratum granulosum* and the eleidin granules RANVIER (*Arch. Anat. Micr.*, iii, 1899, p. 1) hardens with alcohol, stains with picro-carmine, and treats with lime-water. The cells swell and show up the granules, which do not change. See *loc. cit.*, other methods for the study of skin.

BUZZI (see *Encycl. mik. Technik.*, article "Haut") stains sections for a few minutes in a watchglassful of water with 2 to 3 drops of 1 per cent. Congo red. Similarly WEIDENREICH, *Arch. mik. Anat.*, lvii, 1901, p. 583. Other authors recommend nigrosin, or Wasserblau, or orcein.

665. Horn, Hair, and Nails.—The elements of hairs and nails may be isolated by prolonged maceration in 40 per cent. potash solution, or by heating with concentrated sulphuric acid. See also VON NATHUSIUS, *Zool. Anz.*, xv, 1892, p. 395.

Horny tissues stain well in safranin or gentian violet (REINKE, *Arch. f. mik. Anat.*, xxx, 1887, p. 183; ERNST, *ibid.*, xlvii, 1896, p. 669; RABL, *ibid.*, xlviii, 1896, p. 489).

Numerous other methods are given in the *Encycl. mik. Technik.*, article "Haut."

666. Skin-nerves and Nerve-endings.—Impregnate with gold chloride. See Chap. XVII, especially § 374.

667. Tactile Corpuscles. See §§ 373–375.—Gold methods are indicated. See also Ranvier, *Traité*, p. 919; Langerhans, *Arch. mik. Anat.*, 1873, p. 730; Kultschizky, *ibid.*, 1884, p. 358; and Smirnow, *Intern. Monatsschr. f. Anat.*, etc., x, 1893, p. 241. (This observer recommends, beside the gold method of Löwit, the rapid bichromate of silver method of Golgi.)

668. Corpuscles of Herbst and Corpuscles of Grandry.—Dogiel (*Arch. Anat. u. Entwickel.*, 1891, p. 182) prefers the methylen blue method. Four per cent. solution of methylen blue, warmed to 40° C., is injected into blood-vessels of the heads of ducks or geese; pieces of skin are removed from the beaks, sectioned in pith, and the sections brought on to slides and moistened with aqueous or vitreous humour from the animal, and left for a few minutes exposed to the air (it is well to add to the aqueous or vitreous humour a few drops of $\frac{1}{16}$ per cent. methylen blue solution). After about ten to thirty minutes the nerve-endings are seen to be stained, and the sections are then brought into picrate of ammonia, and treated as described § 351. Geberg (*Intern. Monatsschr. Anat.*, x, 1893, p. 205) has also employed this method. He has also made use of simple osmic acid, and of a method of Arnstein, § 377, according to which pieces of skin are put for twenty-four hours into lime-water, the horny layer removed, the pieces treated for five minutes with 0·25 per cent. gold chloride, reduced in water, and the precipitate that forms on them removed by putting into 0·25 per cent. cyanide of potassium and brushing. See also Dogiel, § 771.

669. Corpuscles of Meissner and of Krause (Cornea and Conjunctiva Bulbi and Palpebrarum).—Dogiel (*Arch. f. mik. Anat.*, xxxvii, 1891, p. 602, and xliv, 1894, p. 15) employs the methylen blue method; for details see *previous editions.*

See also Longworth's methods, *Arch. mik. Anat.*, 1875, p. 655.

670. Similar Objects.—Papillæ Foliatæ of the Rabbit, HERMANN, see *Zeit. wiss. Mik.*, v, 1888, p. 524; ARNSTEIN, *ibid.*, xiii, 1897, p. 240. **Olfactive Organs of Vertebrates** (DOGIEL, *Arch. mik. Anat.*, 1887, p. 74). **Organs of a "Sixth Sense" in Amphibia** (MITROPHANOW).—See *Zeit. wiss. Mik.*, v, 1888, p. 513 (details as to staining with "Wasserblau," for which see also *Biol. Centralb.*, vii, 1887, p. 175). **Nerve-endings in Tongue of Frog** (FAJERSTAIN [FEUERSTEIN], *Arch. de Zool. expér. et gén.*, vii, 1889, p. 705. **Tongue of Rabbit,** VON LENHOSSÉK, *Zeit. wiss. Mik.*, xi, 1894, p. 377 (Ramón y Cajal's double Golgi-method).

671. Cornea.—There are three chief methods for the study of the corneal tissue—the methylen blue method, the silver method, and the gold method.

For the *methylen blue method* see particularly § 353.

Negative images of the corneal cells are easily obtained by the dry *silver method* (KLEIN). The conjunctival epithelium should be removed by brushing from a living cornea, and the corneal surface well rubbed with a piece of lunar caustic. After half an hour the cornea may be detached and examined in distilled water.

In order to obtain *positive* images of the fixed cells the simplest plan (RANVIER) is to macerate a cornea that has been prepared as above for two or three days in distilled water. There takes place a secondary impregnation, by which the cells are brought out with admirable precision.

The same result may be obtained by cauterising the cornea of a living animal as above, but allowing it to remain on the living animal for two or three days before dissecting it out, or by treating a negatively impregnated cornea with weak salt solution or weak solution of hydrochloric acid (HIS).

But the best positive images are those furnished by *gold chloride.* RANVIER prefers his lemon-juice method (§ 375). It is important that the cornea should *not remain too long in the gold solution,* or the nerves alone will be well impregnated.

RANVIER also recommends this method as the best for the study of the nerves.

See also ROLLETT, in Stricker's *Handb.*, pp. 1102, 1115, or *previous editions*; TARTUFERI, *Anat. Anz.*, v, 1890, p. 524, or *previous editions*; CIACCIO, *Arch. ital. Biol.*, iii, p. 75; and RENAUT, *C. R. Acad. Sc.*, 1880, p. 137.

672. Crystalline.—GEBHARDT (*Zeit. wiss. Mik.*, xiii, 1896, p. 306) hardens the lens for one or two days in 4 to 10 per cent. formalin; it is then easily dissociated with needles into its fibres. See also § 115.

RABL (*Zeit. wiss. Zool.*, lxv, 1898, p. 272) fixes the enucleated eye for half an hour in his platinum chloride or picro-sublimate, §§ 81 and 75, divides it at the equator, and puts the anterior half back for twenty-four hours into the fixative.

For **Maceration** you may use sulphuric acid, § 549.

CHAPTER XXVIII.

MUSCLE AND TENDON (NERVE-ENDINGS).

Striated Muscle.

673. Muscle-cells.—For the study of these and allied subjects see, *inter alia*, BEHRENS, KOSSEL, und SCHIEFFERDECKER, *Das Mikroskop*, etc., vol. ii, pp. 154—161; also for the application of the gold method to the study of muscle-cells, SCHÄFER, *Proc. Roy. Soc.*, xlix, 1891, p. 280; or *Journ. Roy. Mic. Soc.*, 1891, p. 683.

Iron hæmatoxylin gives very fine images of striped muscle. See also § 328.

For dissociation methods see §§ 531, 545, 546, 552.

To isolate the sarcolemma SOLGER (*Zeit. wiss. Mik.*, vi, 1889, p. 189) teases fresh muscle in saturated solution of ammonium carbonate.

674. Nerve-endings—the Methylen Blue Method.—BIEDERMANN'S procedure for the muscles of *Astacus* has been indicated in § 350 (see also *Zeit. wiss. Mik.*, vi, 1889, p. 65). After impregnating as there directed the carapace should be opened, and the muscles exposed to the air in a roomy moist chamber for from two to six hours, in order that the stain may differentiate. The abdominal and caudal muscles are those which give the best results.

For *Hydrophilus piceus*, BIEDERMANN proceeded by injecting 0·5 c.c. of methylen blue solution between the ultimate and penultimate abdominal rings, in the ventral furrow, and keeping the animals alive in water for three to four hours. After this time the thorax should be opened by two lateral incisions, and the muscles of the first pair of legs (which are the most suitable) removed and exposed to the air for three or four hours in a moist chamber, and finally examined in salt solution.

GERLACH (*Sitzb. k. math.-phys. Cl. k. bayer. Akad. Wiss.*

München, 1889, ii, p. 125; *Zeit. wiss. Mik.*, vii, 1890, p. 220) injected frogs, either through the abdominal vein or through the aorta, with 4 to 5 c.c. of a 1 : 400 solution in 1 per cent. salt solution, and examined pieces of muscle (perferably the head and eye muscles) in serum of the animal, afterwards fixing the preparations with picrate of ammonia and mounting in glycerin jelly.

The procedure of Dogiel has been given § 350.

675. Nerve-endings—the Gold Method.—Fischer (*Arch. mik. Anat.*, 1876, p. 365) used the method of Löwit, § 373.

Biedermann in the paper quoted in the last section recommends for *Astacus* a similar procedure, the preliminary treatment with formic acid being omitted, and the muscles being put for a couple of days into glycerin after reduction in the acid.

Ranvier (*Traité*, p. 813) finds that for the study of the motor terminations of Vertebrates the best method is his lemon-juice process (§ 375). The delicate elements of the arborescence of Kühne are better preserved by this method than by the simple method of Löwit.

See also the methods of Apáthy, §§ 377, 380.

676. Nerve-endings—the Silver Method.—Ranvier employs it as follows (*ibid.*, p. 810): Portions of muscle (gastrocnemius of frog) having been very carefully teased out in fresh serum, are treated for ten or twenty seconds with nitrate of silver solution of 2 to 3 per 1000, and exposed to bright light (direct sunlight is best) in distilled water. As soon as they have become black or brown they are brought into 1 per cent. acetic acid, where they remain until they have swelled up to their normal dimensions. They are then examined in a mixture of equal parts of glycerin and water.

This process gives *negative* images, the muscular substance is stained brown, except in the parts where it is protected by the nervous arborescence, which itself remains unstained. The gold process gives *positive* images, the nervous structures being stained dark violet.

677. Nerve-endings—the Bichromate of Silver Method.—The *rapid* method of Golgi has been applied by Ramón y Cajal to the study of the terminations of nerves and of tracheæ in the muscles of insects. See *Zeit. wiss. Mik.*, vii, 1890, p. 332, or *fourth edition*.

Tendon.

678. Corpuscles of Golgi (RANVIER, *Traité*, p. 929).—Take the tendon of the anterior and superior insertion of the gemini muscles of the rabbit. Free it as far as possible from adherent muscle-fibres. Treat it according to the formic acid and gold method (§ 374), and after reduction of the gold scrape the tendon with a fine scalpel, in order to remove the muscle-fibres that mask the "musculo-tendinous organs."

679. Corpuscles of Golgi (in the tendons of the motores bulbi oculi) (VON MARCHI's methods, *Archivio per le Scienze Mediche*, vol. v, No. 15).—The enucleated eyes, together with their muscles, were put for not less than three days into 2 per cent. bichromate of potash. The muscles and tendons were then carefully dissected out, stained with gold chloride and osmic acid (GOLGI's method), and by the method of MANFREDI, § 377.

Mount all these preparations in glycerin (balsam clears too greatly). The methods only succeed completely during fine sunny weather.

See also RUFFINI (*Atti R. Acc. Lincei Roma Rend.* [5], i, 1892, p. 442; *Zeit. wiss. Mik.*, ix, 1892, p. 237), who recommends the method of Fischer.

680. Corpuscles of Golgi (CIACCIO, *Mem. R. Acc. Sci. Bologna* [4], t. x, 1890, p. 301; *Zeit. wiss. Mik.*, vii, 1891, p. 507). —For Amphibia the usual gold methods are not satisfactory, because the ground-substance of the tendon takes the stain at the same time as the nerve-endings. Pieces of tendon should be put into 0·1 per cent. hydrochloric acid or 0·2 per cent. acetic acid until quite transparent. They should then be put for five minutes into a mixture of 0·1 per cent. gold chloride and 0·1 per cent. potassium chloride. After that they are put back into the acetic acid, and remain there for a day in the dark, and for two or three hours more in the sunlight. When they have become somewhat violet they are put for a day into 0·1 per cent. osmic acid, and finally mounted in Price's glycerin acidulated with 0·5 per cent. of acetic or formic acid.

Smooth Muscle.

681. Tests for Smooth Muscle.—Picro-säurefuchsin, § 309, stains muscle yellow, connective tissue red.

Picro-nigrosin, § 332, stains muscle yellowish, connective tissue blue.

Unna (*Encycl. mik. Technik.*, article "Kollagen") stains for twenty-four hours in orcein 1 part, Wasserblau 0·25, alcohol 60, glycerin 10, water 30, which gives muscle in a mixed tone, collagen blue, elastin reddish. You may dehydrate with either neutral or acid alcohol, the latter giving more contrast. See also next §.

682. Smooth Muscle, Specific Stain for (Unna, *Monatssch. prakt. Dermatol.*, xix, 1894, p. 533; *Zeit. wiss. Mik.*, xii, 1895, p. 243).—Sections stained for ten minutes in polychromatic methylen blue solution, rinsed in water, and brought for ten minutes into 1 per cent. solution of red prussiate of potash, then into alcohol acidified with 1 per cent. of hydrochloric acid for about ten minutes (until the collagen ground comes out white). Absolute alcohol, essence, balsam.

In the same place see also another stain with acid orcein, hæmatein, Säurefuchsin, and picric acid.

683. Smooth Muscle Isolation of Fibres.

Gage's methods, see §§ 531, 552, and 545.

Möbius, muscle of *Cardium*, see § 544.

Ballowitz, muscle of Cephalopoda, see *Arch. mik. Anat.*, xxxix, 1892, p. 291.

Schultz (*Arch. Anat. Phys., Phys. Abth.*, 1895–6, p. 521) puts smooth muscle of Vertebrates for twenty-four hours into 10 per cent. nitric acid, rinses with water, and brings pieces for six to eight days (in the dark at first) into a mixture of equal parts of $\frac{1}{20}$ per cent. osmic acid and $\frac{1}{5}$ per cent. acetic acid, teases, and mounts in glycerin.

For smooth muscle of Vermes, see Apáthy, *Zeit. f. wiss. Mik.*, x, 1893, pp. 36, 319.

684. Iris (DOGIEL, *Arch. mik. Anat.*, 1886, p. 403).—An enucleated eye is divided into halves, and the anterior one with the iris brought for some days into a mixture of two parts one third alcohol and one part 0·5 per cent. acetic acid. The iris can then be isolated, and split from the edge into an anterior and posterior plate, and these stained according to the usual methods.

See also KOGANEI, *Arch. mik. Anat.*, 1885, p. 1; CANFIELD, *ibid.*, 1886, p. 121; and DOSTOIEWSKY, *ibid.*, p. 91.

685. Bladder of Frog, Innervation of (WOLFF, *Arch. f. mik. Anat.*, 1881, p. 362).—A frog is killed and a solution of gold chloride of 1 : 20,000 injected into the bladder through the anus. (If the injection flows out on removal of the syringe, tie the frog's thighs together.) Now open the frog, dissect away the attachments of the bladder, ligature the intestine above the bladder, and cut away the abdomen so as to have in one piece bladder, rectum, and hind legs. (All this time the bladder must be kept moist with weak gold solution.) The bladder and the rest are now put into gold solution of 1 : 2000 for four hours; the bladder is then excised, slit open, and pinned (with hedgehog spines) on to a cork (outside downwards). Place it under running water until all the epithelium is washed away. Use a camel's-hair brush if necessary. Put for twenty-four hours into gold solution of 1 : 6000. Wash in pure water, and put away in the dark "for some time" in acidulated water, and finally reduce in fresh water in common daylight. The muscles should be pale blue-red; medullated nerves dark blue-red; sympathetic nerves and ganglia carmine-red. RANVIER (*Traité*, p. 854) recommends his two gold processes, §§ 374, 375, the liquids being injected as above.

See also § 377, BERNHEIM.

GRÜNSTEIN (*Arch. Mik. Anat.*, 1899, p. 1) injects 1 per cent. methylen blue in normal salt solution through the *vena abdominalis*, and after twenty to thirty minutes excises the bladder and exposes to the air. When the stain is obtained, it is fixed with picrate of ammonia and mounted in glycerin with the same (§ 351).

686. Stomach of Triton (see STILLING and PFITZNER, in *Arch. mik. Anat.*, 1886, p. 396).

CHAPTER XXIX.

CONNECTIVE TISSUES.

Connective Tissue.

687. General Stains for Connective Tissue.—It is frequently difficult to distinguish between connective tissue, elastic tissue, and smooth muscle in preparations. All three are *normally* acidophilous. Collagen, the distinctive element of connective tissue, absolutely requires "acid" dyes for the production of a permanent stain, whilst elastic tissue and muscle will also fix "basic" dyes. Collagen has a special affinity for Säurefuchsin and Wasserblau, the former of these forming the principal ingredient in mixtures for the differentiation of these tissues. Elastin has a strong affinity for acid orcein, whilst muscle has no special affinity for either, but stains energetically with picric acid.

Picro-säurefuchsin, § 309, is much used and very convenient as a general differentiating stain, but not to be recommended for cytological detail.

Ehrlich-Biondi mixture, § 306, gives connective tissue red, but smooth muscle redder still.

Unna's Wasserblau-orcein for distinguishing connective tissue and muscle has been given § 681.

Freeborn (*Amer. Mon. Mic. Journ.*, 1888, p. 231) recommends (for sections) *picro-nigrosin,* made by mixing 5 c.c. of 1 per cent. aqueous solution of nigrosin with 45 c.c. of aqueous solution of picric acid. Stain for three to five minutes, wash with water, and mount in balsam. Connective tissue blue, nuclei blackish, the rest yellowish.

Ramón y Cajal's *picro-indigo-carmine,* § 396, gives connective-tissue fibres dark blue, with red nuclei.

S. Mayer (*Sitzb. k. Akad. Wiss.*, lxxxv, 1882, p. 69) recommends for staining *fresh* tissue Violet B, § 337. Elastic fibres and smooth muscle also stain, but of different tints.

For Ranvier's method of artificial œdemata for the study of areolar tissue, see his *Traité*, p. 329.

688. Benecke's **Stain for Fibrils** (*Verh. d. anat. Ges.*, vii Vers., 1893, p. 165, is essentially that of Kromayer, § 662.

See also Kromayer, *Dermat. Zeit.*, iii, 1896, p. 263; *Zeit. wiss. Mik.*, xiv, 1897, p. 56.

689. Unna's **Orcein Method** (*Monatssch. prakt. Dermatol.*, xviii, 1894, p. 509; *Zeit. wiss. mik.*, xi, 1894, p. 518). Sections are stained for five minutes in Grübler's strong solution of polychromatic methylen blue. They are then brought for fifteen minutes into a neutral 1 per cent. solution of orcein in absolute alcohol, rinsed in pure alcohol, cleared in bergamot oil, and mounted. Nuclei blue, collagenous ground-substance dark red, granules of Mastzellen carmine red, protoplasm of Plasmazellen bluish, muscle bluish, elastic fibres sometimes dark red. Material may be fixed in almost any way except with nitric or picric acid, formol, or liquids of Müller and Hermann.

690. Unna's **Methylen-blue + Säurefuchsin** (Unna, in *Encycl. mik. Technik*, 1903, art. "Kollagen," where see further details and other methods). Stain for 2–5 minutes in polychrome methylen blue solution (Grübler). Wash and stain for 10–15 minutes in "(0·5 %) Säurefuchsin + (33 %) tannin-mixture (Grübler)". Water, alcohol, essence, balsam. Collagen, protoplasm, and muscle red, nuclei and keratin, blue. On Flemming material, elastin blue. Liquids of Hermann and Erlicki, formol and copper fixatives incompatible.

691. Unna's **Safranin + Wasserblau** (*ibid.*). Ten minutes in 1 % safranin. Wash. Ten to 15 minutes in "(1 %) Wasserblau + (33 %)—tannin mixture." Wash. Stains in opposite colours to the last. Formol and liquid of Hermann contra-indicated for fixing.

692. Flemming's Orange Method, § 310, is said to give a very sharp differentiation of *developing fibrils*. For this object see also two methods of Unna, *op. cit.*, § 690 (p. 694).

Elastic Tissue.

693. Elastic Tissue, Generalities.—Elastic fibres have a great affinity for osmium, staining with much more rapidity than most other tissue elements. They are not changed by caustic soda or potash. They are *normally* acidophilous, but are easily rendered *artificially* basophilous by means of chromic acid or other mordants, and then stain with great energy with basic dyes (see § 218). Hence a group of stains of which those of Lustgarten and Martinotti are types. They have a natural affinity for orcein, whence stains of the Taenzer-Unna type.

For a review of the older methods of Balzer, Unna, Lustgarten, and Herxheimer, see the paper by G. Martinotti in *Zeit. wiss. Mik.*, iv, 1887, p. 31; also *Encycl. mik. Technik.*, art. "Elastin."

694. Victoria Blue (Lustgarten). See § 301.

695. Safranin (G. Martinotti, *loc. cit.*, § 693).—Fix in a chromic liquid, wash, stain for forty-eight hours in strong (5 per cent. Pfitzner's) solution of safranin, wash, dehydrate, clear, and mount in balsam. Elastic fibres are stained an intense black, the rest of the preparation showing the usual characters of a safranin stain.

The staining will be performed quicker if it be done at the temperature of an incubating stove (Griesbach, *ibid.*, iv, 1887, p. 442). See also Ferria (*ibid.*, v, 1888, p. 342).

Another safranin method is that of Mibelli, see *Mon. zool. Italiano*, 1, p. 17, or *Zeit. wiss. Mik.*, vii, 1890, p. 225 (the report in *Journ. Roy. Mic. Soc.*, 1890, p. 803, is vitiated by a misprint). Other basic dyes have been recommended.

696. Kresofuchsin (Röthig, see § 301).—Dissolve 0·5 g. Kresofuchsin in 100 c.c. alcohol, and add 3 c.c. of hydrochloric acid. Mix 40 c.c. of this solution (which contains undissolved substance) with 24 c.c. of alcohol, and add xxxii drops of saturated aqueous picric acid diluted with 2 vols. of water. Stain for two hours or more, differentiate in alcohol and pass through xylol into balsam. Nuclei may be counterstained. See also § 698.

697. Orcein.—This method is due to Taenzer, and as modified by Unna is known as the Taenzer-Unna method, see *third edition*, or *Monatssch. prakt. Dermatol.*, xii, 1891, p. 394 (*Zeit. wiss. Mik.*, ix, 1892, p. 94).

Unna's **Modified Orcein Method** (*Monatssch. prakt. Dermatol.*, xix, 1894, p. 397; *Zeit. wiss Mik.*, xii, 1895, p. 240).—The following solution is made: Grübler's orcein 1 part, hydrochloric acid 1 part, absolute alcohol 100 parts. The sections are put into a porcelain capsule with just enough of the stain to cover them, and the whole is warmed to about 30° C. After ten to fifteen minutes the stain becomes quite thick, owing to evaporation of the alcohol. The sections are then well rinsed in alcohol, cleared, and mounted. Elastin dark brown, collagen light brown. I believe the warming is not necessary. Müller or Flemming material may be used as well as alcohol material, and you may counterstain if desired.

See also Merk, *Sitz. Akad. Wiss. Wien*, cviii, 1899, p. 335 (*Zeit. wiss. Mik.*, xvii, 1900, p. 73); Pranter (*ibid.*, xix, 1903, p. 361; he takes 2 per cent. of nitric acid instead of the hydrochloric, and stains six to twenty-four hours); Wolff (*ibid.*, p. 488); and the article "Elastin" in *Encycl. mik. Technik.*, as also the article "Kollagen," p. 695.

698. Weigert's **Fuchsin-Resorcin Method** (*Zeit. wiss. Mik.*, xvi, 1899, p. 81).—1 per cent. of basic fuchsin and 2 per cent. of resorcin (or of carbolic acid) are dissolved in water. 200 c.c. of the solution are raised to boiling point in a capsule, and 25 c.c. of *Liquor ferri sesquichlorati* P. G. are added, and the whole is boiled, with stirring, for two to five minutes more. A precipitate is formed. After cooling the liquid is filtered, and the precipitate which remains on the filter is brought back into the capsule, and there boiled with 200 c.c. of 94 per cent. alcohol. Allow to cool, filter, make up the filtrate to 200 c.c. with alcohol, and add 4 c.c. of hydrochloric acid.

Stain sections (of material fixed in any way) for twenty minutes to an hour, wash with alcohol, clear with xylol (not with an essence). Elastic fibres dark blue on a light ground. Nuclei generally unstained; they may be after-stained. Cartilage is also stained.

Mayer (*Grundzüge*, p. 458) finds it advantageous to add a very little chloride of iron to the filtrate (so also for Kresofuchsin, § 696).

Minervini (*Zeit. wiss. Mik.*, xviii, 1901, p. 161) gives a variant with safranin instead of fuchsin.

See also PRANTER, *ibid.*, xix, 1903, p. 361, and B. FISCHER, *Virchow's Arch.*, clxx, 1902, p. 285, or *Zeit. wiss. Mik.*, xx, 1903, p. 40 (chemistry of the dyes obtained by these processes, which he calls "Fuchselin," "Safranelin," etc.).

699. Other Methods for Elastic Tissue:

For the elastic tissue of the skin see PASSARGE and KRÖSING, *Dermat. Stud.*, xviii, 1894.

See also for staining and dissociation AGABABOW, *Arch. mik. Anat.*, l, 1897, p. 566, *et. seq.*

For C. MARTINOTTI'S silver impregnation see *Zeit. wiss. Mik.*, v, 1888, p. 521, or *Arch. Ital. Biol.*, xi, 1889, p. 257.

SCHUMACHER (*Arch. mik. Anat.*, lv, 1899, p. 151) has had good results (for the spleen) with a mixture of 1 part 1 per cent. nigrosin and 9 parts saturated aqueous picric acid.

See also § 731.

See also ZENTHOEFER, in Unna's *Dermatol. Studien*, 1892, or *Zeit. wiss. Mik.*, ix, 1893, p. 509; KÖPPEN, *ibid.*, vi, 1889, p. 473; and vi, 1890. p. 22, or third edition; BURCI, *Journ. Roy. Mic. Soc.*, 1891, p. 831, and 1892, p. 292, or third edition; HANSENN, Virchow's *Archiv*, cxxxvii, 1894, p. 25; *Zeit. wiss. Mik.*, xi, 1894, p. 383; KULTSCHIZKY, *ibid.*, xiii, 1896, p. 74, or the original, *Arch. mik. Anat.*, xlvi, 1895, p. 673; GÜNTHER, *Zeit. wiss. mik.*, xiii, 1896, p. 230; SCHIEFFERDECKER, *ibid.*, p. 302; TRIEPEL, *ibid.*, xiv, 1897, p. 31; LOISEL, *Journ. de l'Anat. et de la Phys.*, xxxiii, 1897, p. 134; GARDNER, *Biol. Centralb.*, xvii, 1897, p. 398; LIVINI, *Monitore Zool. Ital.*, vii, 1896, p. 45 (*Journ. Roy. Mic. Soc.*, 1899, p. 455).

Plasma Cells.

700. Plasma Cells and "Mastzellen"; Generalities.—Plasma cells, of which "Mastzellen" are a sub-species, are cells found in or along with connective tissue, and distinguished by their hypertrophied and very granular cytoplasm and poorly-staining nucleus. The granules are highly basophilous, much more so than the nuclei; they stain with special energy with basic anilins, and mostly metachromatically. They do not, however, stain with pure methyl green. The nuclei either do not stain at all or not in the normal way, except with pure methyl green.

The granules (see UNNA in *Encycl. mik. Tech.*, p. 1119, and EHRLICH, *Virchow's Arch.*, clxxv, 1904, p. 198) have a great affinity for metallic salts and tannic acids, and—according to these authors—their staining reactions are greatly altered by fixatives containing them. Material should therefore be fixed in chemically pure absolute alcohol and sectioned in celloidin. Care should be taken to avoid contamination of the liquids by tannin; corks, and supports for imbedding, should be soaked for some hours before use in 2 per cent. carbonate of soda.

701. Ehrlich's **Original Method for Mastzellen** (*Arch. mik. Anat.*, xii, 1876, p. 263).—Tissues are placed for at least twelve hours in a staining fluid composed of—

Absolute alcohol . . .	50 c.c.
Water	100 c.c.
Acid. acet. glacial . .	12½ c.c.

—to which has been added enough dahlia to give an almost saturated solution. After staining, they are transferred to alcohol, which washes out the stain from all but the plasma cells, and may then be mounted in resin-turpentine solution.

In a similar way (but with only 7½ parts of acid) may be employed—primula, iodine violet, methyl violet, purpurin, safranin, fuchsin; of these, methyl violet gives the best results.

See also Schiefferdecker and Kossel's *Gewebelehre*, p. 329.

702. **Plasma Cells** (Nordmann, *Beitr. z. Kenntniss d. Mastzellen, Inauguraldiss.*, Helmstedt, 1884).—A concentrated solution of vesuvin containing 4 to 5 per cent. of hydrochloric acid. Sections should remain for a few minutes in it, and then be dehydrated with absolute alcohol.

703. **Plasma Cells,** Unna's **Latest Methods** (Unna, in *Encycl. mik. Tech.*, 1903, p. 1120).

A.—For Large Plasma Cells.

(1) Ten minutes in Grübler's polychrome methylen blue solution, wash and drain. Fifteen minutes in 1 per cent. orcein solution (Grübler), without acid; absolute alcohol, so long as methylen blue comes away abundantly; bergamot oil, balsam.

(2) Methylen blue as above, 2 minutes. Wash well. Then two minutes in glycerin-ether mixture* (Grübler) diluted with 4 vols. of water. Wash thoroughly (2 to 5 minutes); absolute alcohol, bergamot oil, balsam.

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* Glycerin ether, $C_6H_{10}O_3$, is a glycerin anhydride. It is a differentiating agent for basic dyes. The glycerin-ether mixture in question contains alcohol and glycerin, and can be obtained from Grübler.

(3) Modification of a method of PAPPENHEIM (*Virchow's Arch.*, clxiv, 1901, p. 111). Ten minutes in the warm, 20° to 40° C., in Grübler's carbol-pyronin-methyl-green mixture. *Cool rapidly*, by plunging the recipient containing the tissues into cold water. Remove the tissues with a platinum wire and rinse. Absolute alcohol, bergamot oil, balsam.

B.—For small Plasma Cells.

(4) As No. 2, *supra*, but only half a minute in the glycerin-ether.

(5) After removal of the celloidin from the sections with alcohol and ether, five minutes in polychrome methylen blue, wash, dry with blotting-paper, dehydrate (about a minute) in a mixture of two parts alcohol to three of xylol, then one minute in xylol; then 5 to 10 minutes in alum-anilin (prepared by allowing anilin oil to stand over a layer of powdered alum a couple of fingers deep); xylol, balsam.

(6) As No. 3, *supra*, after a foregoing stain of two minutes in polychrome methylen blue.

704. Plasma Cells, EHRLICH'S **Latest Methods** (*Virchow's Arch.*, clxxv, 1904, p. 198).

(1) As No. 2, last §. Two to five minutes in the methylen blue, wash till no more colour comes away (10 to 30 min.), then 5 to 10 in dilute glycerin-ether (15 to 20 drops in a watch-glass of water), wash very thoroughly.

(2) As No. 5, last §, dehydrating in successive mixtures of 4 parts alcohol to 3 of xylol, 3 of alcohol to 3 of xylol, 2 of alcohol to 3 of xylol, half a minute in each.

(3) As No. 3, last §, taking great care to *cool quickly*.

705. Mastzellen, UNNA'S **Latest Methods** (*Encycl. mik. Tech.*, 1903, p. 793).—(1) Stain three hours to over night in polychrome methylen blue with a knife-point-ful of alum to a watch-glass of the stain, rinse; alcohol, oil, balsam. (2) Stain in polychrome methylen blue quarter of an hour, rinse, then ten minutes in glycerin-ether, § 703, wash thoroughly, alcohol, oil, balsam.

These methods give a specific metachromatic stain of Mastzellen on a light ground. See also *loc. cit.*, two other methods demonstrating plasma cells at the same time.

706. Plasma Cells and Mastzellen, Other Methods.—See *previous editions*, or UNNA, *Zeit. wiss. Mik.*, viii, 1892, p. 475; *Monatssch. f. prakt Dermatol.*, xii, 1891, p. 296; *Zeit. wiss. Mik.*, ix, 1892, p. 92, and *Monatssch. f. prakt. Dermatol.*, xix, 1894, p. 225; *Zeit. wiss. Mik.*, xii, 1895, p. 58; also VAN DER SPEK and UNNA, *loc. cit.*, xiii, 1891, p. 364; *Zeit. wiss. Mik.*, xi, 1892, p. 89; BERGONZINI (*Anat. Anz.*, vi, 1891, pp. 595–600; JADASSOHN, *Arch. f. Dermatol. u. Syphilis*, Ergänzungsheft 1, 1892, p. 58; *Zeit. wiss. Mik.*, ix, 1892, p. 226; VON MARSCHALKÒ, *Arch. f. Dermatol. u. Syphil.*, xxx, 1895, p. 3; *Zeit. wiss. Mik.*, xii, 1885, p. 64; KROMPECHER, *ibid.*, xv, 1899, p. 458; RANVIER, *C. R. Acad. des Sci.*, 1890 (his **Clasmatocytes**: fix with osmic acid, stain with methyl violet 5 B); PAPPENHEIM, *Virchow's Arch.*, clxiv, 1901, p. 111, and clxvi, 1901, p. 424, or *Zeit. wiss. Mik.*, xix, 1902, pp. 97, 98 (the stain mentioned § 703).

Fat.

707. Fat, Removal of.—DEKHUYSEN and FLEMMING (*Zeit. wiss. Mik.*, 1889, pp. 39, 178) find that fat that has been stained black by treatment with chromo-aceto-osmic acid is dissolved in the course of a few hours in turpentine, xylol, ether, or creasote, and more slowly if it has been blackened with pure osmic acid.

708. Fat and Lecithin.—According to LOISEL (*C. R. Soc. Biol.*, lv, 1903, p. 703) lecithin is much less soluble than neutral fats in turpentine or ether, and in general much more stainable. To demonstrate and distinguish lecithin, he advises as follows: After fixation, mordant the tissues with alum; leave them as little as possible in alcohol; dehydrate with acetone, ether, or benzin; stain with hæmatoxylin, gentian, methyl green, toluidin blue, Säurefuchsin or orange G, which stain lecithin strongly, whilst leaving fat unstained.

709. Preservation of Fat.—Osmicated fat can generally be mounted in balsam without special precaution. For delicate fats it may suffice to avoid absolute alcohol and essences as much as possible, and mount direct in alcohol balsam (§ 452), or clear with cedar oil, which has little solvent action. For very delicate fats it may be necessary to avoid alcohol of more than 70 per cent., or avoid it altogether, and mount in glycerin or levulose.

710. Stains for Fat.—The simplest stain for fat is **osmic acid** (see §§ 39 and 707).

Daddi (*Arch. Ital. Biol.*, xxvi, 1896, p. 143) stains fat in tissues by treating for 5 to 10 minutes with concentrated alcoholic solution of **Sudan III**, washing for the same time with alcohol, mopping up with blotting-paper, and mounting in glycerin. The stain is said to be more selective for fats than that of osmic acid.

Similarly Rieder, see *Zeit. wiss. Mik.*, xv, 1898, p. 211.

The alcohol for making the stain should be of 70 per cent., according to most authors, though Sata (*Beitr. path. Anat.*, xxviii, 1900, p. 461; *Zeit. wiss. Mik.*, xviii, 1901, p. 67) employs 96 per cent. Rosenthal (*ibid.*, xix, p. 469; *Verh. path. Ges.*, Sept. 1899, p. 440) insists that the washing-out be done with alcohol of exactly 50 per cent. Michaelis (*Virchow's Arch.*, clxiv, 1901, p. 263) recommends **Scharlach R** (syn. "Fettponceau"). Stain for fifteen to thirty minutes in a saturated solution in 70 per cent. alcohol, and mount in glycerin or levulose.

Other authors also commend this stain. Herxheimer (*Deutsche med. Wochenschr.*, xxvii, 1901, p. 607; *Zeit. wiss. Mik.*, xix, 1902, p. 66) makes a solution of 70 parts of absolute alcohol, 10 of water, 20 of 10 per cent. caustic soda, and Scharlach R to saturation. This makes a stronger solution, and stains in a couple of minutes. Wash out with alcohol of 70 per cent.

With either solution the staining must be done in a covered vessel or the stain will precipitate.

Later (*Centralb. allg. Path.*, xiv, 1903, p. 841; *Zeit. wiss. Mik.*, xxi, 1904, p. 57) Herxheimer recommends a saturated solution of the dye in a mixture of equal parts of acetone and 70 per cent. alcohol.

He also (*Deutsche med. Wochenschr.*, xxvii, 1901, p. 607; *Zeit. wiss. Mik.*, xix, 1902, p. 67) has had very fine results by staining for twenty minutes in a saturated solution of **Indophenol** in 70 per cent. alcohol.

Mollison (*Zeit. wiss. Zool.*, lxxvii, 1904, p. 529) has had good results by staining gelatin sections for a few minutes in strong extract of **Alkanna** in 96 per cent. alcohol, and mounting in glycerin or syrup.

See also the article "Fett" in *Encycl. mik. Technik.*

Bone.[*]

711. Bone, Non-decalcified, Ranvier (*Traité,* p. 297) points out that when bones are allowed to dry before being put into water for maceration the fat of the medullary canals infiltrates their substance as fast as its water evaporates, and forms fatty spots that prevent good preparations from being obtained.

Bones should be plunged into water as soon as the surrounding soft parts have been removed, and should be divided into lengths with a saw whilst wet. The medulla should then be driven out from the central canal by means of a jet of water; spongy bones should be submitted to hydrotomy as follows: An epiphysis having been removed, together with a small portion of the diaphysis, a piece of caoutchouc tubing is fixed by a ligature on to the cut end of the diaphysis, and the free end of the piece of tubing adapted to a tap through which water flows under pressure.

As soon as the bones, whether compact or spongy, have been freed from their medullary substance they are put to macerate. The maceration should be continued for several months, the liquid being changed from time to time. As soon as all the soft parts are perfectly destroyed, the bones may be left to dry. When dry, they should be of an ivory whiteness, and their surfaces exposed by cutting of a uniform dulness.

Thin sections may then be cut with a saw and prepared by rubbing down with pumice-stone. Compact pumice-stone should be taken and cut in the direction of its fibres. The surface should be moistened with water and the section of bone rubbed down on it with the fingers. When both sides of the sections have been rubbed smooth in this way, another pumice-stone may be taken, the section placed between the two, and the rubbing continued. As soon as the section is thin enough to be almost transparent it is polished by rubbing with water (with the fingers) on a Turkey hone or lithographic stone. Spongy bone should be soaked in gum and dried before rubbing down (but see Von Koch's copal process, *ante*, § 190, and Ehrenbaum's colophonium process, § 191).

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[*] For a minutely detailed review (40 pages, with references to 80 memoirs) of the whole subject of the technique of bone, see the paper of Schaffer in *Zeit. wiss. Mik.*, x, 1893, p. 167, or the article "Knochen und Zähne" in *Encycl. mik. Technik.*

For the process of Weil for bone and teeth see § 193.

Röse (*Anat. Anz.*, vii, 1892, pp. 512–519) follows Koch's process. He penetrates first with a mixture of cedar oil and xylol, then with pure xylol, and imbeds in solution of damar in chloroform or xylol. The method can be combined with Golgi's impregnation.

White (*Journ. Roy. Mic. Soc.*, 1891, p. 307) recommends the following: Sections of osseous or dental tissue should be cut or ground down moderately thin, and soaked in ether for twenty-four hours or more. They should then be put for two or three days into a thin solution of collodion stained with fuchsin (made by dissolving the dye in methylated spirit, adding the requisite quantity of ether, then the pyroxylin). The sections are then put into spirit to harden the collodion. After this they are ground down to the requisite thinness between two plates of old ground glass, with water and pumice powder, and mounted, *surface dry*, in stiff balsam or styrax, care being taken to use as little heat as possible. Lacunæ, canaliculi, and dentinal tubuli are found infiltrated by the coloured collodion.

Matschinsky (*Arch. mik. Anat.*, xxxix, 1892, p. 151, and xlvi, 1895, p. 290), after grinding, impregnates with nitrate of silver.

For a similar method of Ruprecht, see *Zeit. wiss. Mik.*, xiii, 1896, p. 21, wherein see also quoted (p. 23) a method of Zimmermann.

712. Sections of Bones or Teeth showing the Soft Parts.—Nealey (*Amer. Mon. Mic. Journ.*, 1884, p. 142; *Journ. Roy. Mic. Soc.*, 1885, p. 348) says that perfectly *fresh* portions of bone or teeth may be ground with emery on a dentist's lathe, and good sections, with the soft parts *in situ*, obtained in half an hour.

Hopewell-Smith (*Journ. Brit. Dent. Ass.*, xi, 1890, p. 310; *Journ. Roy. Mic. Soc.*, 1890, p. 529) says that for preparing sections of teeth showing odontoblasts *in situ* the best plan is to take embryonic tissues. A lower jaw of an embryonic kitten or pup may be taken, and hardened in solution of Müller followed by alcohol, then cut with a freezing microtome. The knife cuts quite easily the thin cap of semi-calcified dentine and bone.

Weil (*loc. cit.*, § 193) fixes pieces of fresh teeth in sublimate, stains with borax-carmine, brings them through alcohol into chloroform and chloroform-balsam, and after hardening this by heat proceeds to grind as usual (§ 190).

See also Röse, last §.

For the study of the *vessels in teeth*, Lepkowsky (*Anat. Hefte*, viii, 1897, p. 568) injects with Berlin blue, hardens the teeth with a piece of the jaw for one or two days in 50 per cent. formol, decalcifies in 10 per cent. nitric acid (eight to fourteen days, change frequently) and makes celloidin sections.

713. Vivante (*Intern. Monatssch. Anat. u. Phys.*, ix, 1892, p. 398) has made out that thin specimens of bone can be successfully treated by Golgi's bichromate of silver process. He places portions of frontal bone of four to six months calves, which are not more than 3 to 4 mm. thick, for eight days in solution of Müller, then in the osmium bichromate mixture, and then in the silver solution. After impregnation the specimens should be decalcified, which may be done by putting them for twenty days into von Ebner's mixture (§ 574), after which they should be well washed with water and brought into solution of carbonate of soda, and finally imbedded in paraffin. For his quinoleïn blue method see *fourth edition*.

For Underwood's gold process for teeth, and for that of Lepkowski, see *third edition*.

714. Bone, Decalcified (Flemming, *Zeit. wiss. Mik.*, 1886, p. 47).—Sections of decalcified bone are soaked in water, and brought in a drop of water on to a glass plate, where they are spread out flat. The excess of water is removed with blotting-paper, and the sections are covered with another glass plate, to prevent them from rolling. The whole is brought into a plate and covered with alcohol. After the lapse of half an hour the sections have become fixed in the flat position, and may be brought into absolute alcohol without risk of their rolling. To mount them, wash them with fresh alcohol (which may be followed by ether); lay them again flat on glass, and cover them with a double layer of blotting-paper and a somewhat heavy glass plate, and let them dry for a day in the air or in a stove. When they are dry, put a drop of melted balsam on a slide, and let it spread out flat and cool. Prepare a thin glass cover in the same way, put the section on the prepared slide, cover it with the prepared cover, put on a clip, and warm.

By this process sections can be very expeditiously prepared, which show the lacunar system injected with air in quite as instructive a manner as non-decalcified sections.

715. Stains for Cartilage (and Decalcified Bone).—See hereon Schaffer in *Zeit. wiss. Mik.*, v, 1888, p. 1 ; and *Encycl. mik. Technik.*, art. " Knochen."

Kölliker (*Zeit. wiss. Zool.*, xliv, 1886, p. 662) recommends the following for the demonstration of the fibres of Sharpey in decalcified bone. Sections are treated with concentrated acetic acid until they become transparent, and are then put for one quarter to one minute into a concentrated solution of indigo-carmine, then washed in water and mounted in glycerin or balsam. The fibres of Sharpey appear stained of a pale or dark *red*, the remaining bone substance blue.

Zachariadès (*Zeit. wiss. Mik.*, x, 1893, p. 447) has the following : Bone is decalcified by means of picric acid, washed, and put into alcohol and sectioned. The sections are placed on a slide and treated for a few seconds with 1 per cent. solution of osmic acid. They are then stained, either for twenty-four hours in a weak aqueous solution of quinoleïn blue, or for a few minutes in a saturated aqueous solution of safranin. They are then treated on a slide with a drop of 40 per cent. solution of caustic potash, the slide being warmed over a flame until the sections spread out flat. The excess of potash is then removed and the sections are carefully washed with water, covered, and examined. The safranin preparations may be permanently preserved in glycerin containing a small proportion of safranin.

Schaffer's *safranin method* (*Zeit. wiss. Mik.*, v. 1888, p. 17, modified from Bouma, *Centralb. med. Wiss.*, 1883, p. 866).—Sections of decalcified bone are stained for half an hour to one hour in 0·05 per cent. aqueous solution of safranin, washed with water, put for two or three hours in 0·1 per cent. solution of corrosive sublimate, and examined in glycerin, or rinsed with alcohol, cleared *for a long time* in bergamot oil or clove oil, and mounted in xylol balsam.

This is a *double* stain ; cartilage, orange; bone, uncoloured (or green in chromic objects) ; marrow, red.

Schmorl (*Centralb. allg. Path.*, x, 1899, p. 745 ; *Zeit. wiss.*

Mik., xviii, 1901, p. 73) impregnates lacunæ and canaliculi as follows: Material fixed in any way except with sublimate may be used. Decalcify and make celloidin sections. Put into water for at least ten minutes, then into a mixture of 2 c.c. concentrated sol. of Thionin in 50 per cent. alcohol and 10 c.c. water. Stain for ten minutes or more, rinse, and put into saturated aqueous sol. of picric acid for thirty to sixty seconds. Rinse, wash well in alcohol of 70 per cent. till colour no longer comes away freely, then 96 per cent., origanum oil or carbol-xylol, balsam. Matrix yellow, lacunæ and canaliculi brown to black, cells red, fat-cells violet. It may be well to after-stain with Delafield's hæmatoxylin.

Schmorl also describes, *loc. cit.*, a more complicated method with thionin and phosphotungstic or phosphomolybdic acid.

Schaffer also lately (*Centralb. Physiol.*, 1902, H. 20) recommends the methods of Schmorl, or (*Anat. Anz.*, xxiii, 1903, p. 532), for cartilage, simply staining in Thionin of 1 : 50,000 strength. He adds that all stains should be used for cartilage in extreme dilution.

Moll (*Centralb. Physiol.*, xiii, 1899, p. 225; *Zeit. wiss. Mik.*, xvii, 1900, p. 356) stains embryonic cartilage for six to twenty-four hours in orcein 0·5 gr., alcohol 40, water 20, hydrochloric acid 20 drops, and mounts in balsam. Matrix blue, nuclei red.

Bayerl's *method for ossifying cartilage* (*Arch. mik. Anat.*, 1885, p. 35) is as follows: Portions of ossified cartilage are decalcified as directed § 575, cut in paraffin, stained in Merkel's carmine and indigo-carmine mixture, § 394, and mounted in balsam.

Aqueous solution of *benzoazurin* has been commended as a stain for ossifying cartilage by Zschokke, see *Zeit. wiss. Mik.*, x, 1893, p. 381.

A process recommended by Baumgarten has been given, § 397.

Moerner (*Skandinavisches Arch. Physiol.*, i, 1889, p. 216; *Zeit. wiss. Mik.*, vi, 1889, p. 508) gives several stains for tracheal cartilage, chiefly as microchemical tests, for which see *third edition*.

See also a critique of these methods by Wolters in *Arch mik. Anat.*, xxxvii, 1891, p. 492; and on the whole subject of cartilage see Schiefferdecker's *Gewebelehre*, p. 331.

Fusari (*Arch. Ital. Biol.*, xxv, 1896, p. 200; *Zeit. wiss. Mik.*, xiii, 1896, p. 488) makes sections of fresh cartilage, puts them for twenty-four hours into 1 per cent. nitrate of silver, washes, dehydrates, and exposes to the light in balsam.

Cartilaginous Skeletons of embryos (Van Wijhe, *Proc. K. Akad. Wetensch. Amsterdam*, 1902, p. 47) may be studied by staining embryos for a week in a solution of 0·25 g. methylen blue in 100 c.c. of 70 per cent. alcohol, with 1 per cent. of hydrochloric acid. Wash out in alcohol with 1 per cent. of hydrochloric acid until no more colour comes away (about a week), and mount in balsam. The cartilage remains blue, all the other tissues being colourless.

Similarly, Lundvall (*Anat. Anz.*, xxv, 1904, p. 219), using toluidin blue, and instead of mounting in balsam, in which the stain is not well preserved, passing through benzol into a mixture of 4 parts benzol with one of sulphide of carbon, in which the preparations are permanently preserved (in stand-glasses with covers luted with silicate of sodium).

CHAPTER XXX.

BLOOD AND GLANDS.

Blood.

716. Fixing and Preserving Methods.—The school of Ehrlich fix by *heat*. A film of blood is spread on a cover-glass and allowed to dry in the air, and then fixed by passing the cover a few times, three to ten or twenty, through a flame, or by laying it face downwards on a hot plate kept for several minutes or as much as two hours at a temperature at which water not only boils but assumes the spherodial state (110° to 150° C.). For details see Gulland, *Scottish Med. Journ.*, April, 1899, p. 312; Rubinstein, *Zeit. wiss. Mik.*, xiv, 1898, p. 456; Zielina, *ibid.*, p. 463. I can but consider this practice unutterably barbarous, and unsuitable for morphological studies.

In *wet methods* either the blood is *mixed at once*, on being drawn, with some fixing and preserving medium, and studied as a fluid mount, or cover-glass films are prepared and put into a fixing liquid *before the film has had time to dry*.

717. Fixing and Preserving in Bulk.—Most morphologists are agreed that by far the most faithful fixing agent for blood-corpuscles is osmic acid. A drop or two of blood (Biondi recommends two drops exactly) is mixed with 5 c.c. of osmic acid solution, and allowed to remain in it for from one to twenty-four hours. As a rule the osmic acid should be strong —1 to 2 per cent. Fixed specimens may be preserved for use in acetate of potash solution (Max Flesch, *Zeit. wiss. Mik.*, v, 1888, p. 83).

Griesbach also (*op. cit.*, p. 328) prefers osmic acid, which can be combined with certain stains without decomposing them. He mentions methyl green, methyl violet, crystal violet, safranin, eosin, Säurefuchsin, rhodamin, and iodine in potassic iodide.

Rossi (*ibid.*, vi, 1889, p. 475) advises a mixture of equal parts of 1 per cent. osmic acid, water, and strong solution of methyl green, permanent mounts being made by means of glycerin cautiously added.

Ewald (*Zeit. Biol.*, xxxiv, 1897, p. 257) mixes three to four drops of blood of amphibia or reptiles with 10 c.c. of a solution of 0·5 per cent. osmic acid in 0·5 per cent. salt solution (for mammals 0·6 to 0·7 per cent. salt), siphons off the supernatant liquid after twenty-four hours with his capillary siphon (§ 3, p. 4), and substitutes water, alum-carmine, etc., and lastly, 50 per cent. alcohol.

See also Arnold, *Arch. path. Anat.*, cxlviii, 1897, p. 479.

Dekhuysen (*Anat. Anz.*, xix, 1901, p. 536) recommends a mixture of either 3 or 9 vols. of 2 per cent. osmic acid with 1 of 6 per cent. acetic acid, containing $\frac{1}{8}$ per cent. of methylen blue, which he calls "Osmacet."

The mercurial liquids of Pacini (§ 425) used to be considered good. Hayem ("*Du Sang*," etc., Paris, 1889; see also *Zeit. wiss. Mik.*, vi, 1889, p. 335) has the following formula: sublimate 0·5, salt 1, sulphate of soda 5, and water 200. This should be mixed with blood in the proportion of about 1 : 100. Eosin may be added to it. Lowit's formula (*Sitzb. k. Akad. Wiss. Wien*, xcv, 1887, p. 144) consists of 5 c.c. cold saturated sublimate solution, 5 grms. sulphate of soda, 2 grms. salt, and 300 c.c. water. Mosso finds that both of these are too weak in sublimate.

Duboscq (*Arch. Zool. Expér.*, vi, 1899, p. 481) uses (for blood of Chilopoda) a solution of acetic acid, copper acetate, copper chloride, osmic acid, thionin, 1 grm. each, water 400, which, mixed with the blood, fixes and stains in about two minutes.

Formol has lately been used. Marcano (*Arch. de Med. Expér.*, xi, 1899, p. 434) mixes fresh blood with a mixture of 100 parts of sodium sulphate of sp. g. 1·020 and 1 of formol; or with water 85 to 100 parts, sodium chloride 1, and formol 1.

Kizer (*Journ. Roy. Mic. Soc.*, 1900, p. 128) simply mixes

1 drop of blood with 3 of 2 per cent. formalin, and allows to stand for an hour.

Stains can be added to formol or formol mixtures.

Lavdowsky (*Zeit. wiss. Mik.*, x, 1893, p. 4) describes some remarkable results obtained by fixing with 2 per cent. iodic acid and staining with Neu-Victoriagrün, methyl violet 6 B, or gentian violet, a process which is said to reveal the presence of nuclei in elements generally considered to be apyrenematous.

718. Fixing and Preserving in Films (Wet).—Muir (*Journ. of Anat. and Phys.*, xxvi, 1892) makes cover-glass films and drops them into saturated sublimate solution, and after half an hour washes, dehydrates, and passes through xylol into balsam.

Gulland (*Brit. Med. Journ.*, March 13th, 1897; *Scottish Med. Journ.*, April, 1899) makes cover-glass films, and after a few seconds drops them face downwards into a solution of—

Absolute alcohol saturated with eosin .	25 c.c.
Pure ether	25 „
Sublimate in absolute alcohol (2 grms. to 10 c.c.)	5 drops.

After three or four minutes they are washed, stained, and mounted in balsam.

Jenner (*Lancet*, 1899, No. 6, p. 370; *Journ. Roy. Mic. Soc.*, 1899, p. 231) mixes equal parts of 1·2 to 1·25 per cent. water-soluble eosin (Grübler) and 1 per cent. methylen blue, filters after twenty-four hours, washes the precipitate on the filter, dries it, dissolves it in 200 parts of pure methyl alcohol, and puts cover-glass preparations into the solution, in which they are fixed and stained in three minutes.

Or, simply mix 125 c.c. of 0·5 per cent. solution of the eosin in methyl alcohol with 100 c.c. of 0·5 per cent. solution of methylen blue.

Many recent authors fix wet films with formol. Benario (*Deut. med. Wochenschr.*, 1895, p. 572) mixes 1 part of 10 per cent. formol with 9 of alcohol (the mixture must be freshly prepared), and plunges films into it for a minute.

Similarly Wermel (see *Zeit. wiss. Mik.*, xvi, 1899, p. 50), who combines various stains (methylen blue, eosin, gentian, etc.) with the formol.

Edington (*Brit. Med. Journ.*, 1900, p. 19) exposes films for 15 to 30 minutes to vapour of formol under a bell-jar.

Scott (*Journ. of Path. and Bacter.*, vii, 1900, p. 131) exposes films to the vapour for about 5 seconds and drops into absolute alcohol, and after 15 minutes stains and mounts.

719. Stains for Blood.—*Eosin* stains rose-red all parts of blood-corpuscles that contain hæmoglobin (Wissozky, *Arch. mik. Anat.*, 1876, p. 479). Hence a host of stains containing eosin, which is very frequently combined with methylen blue.

Fresh (unfixed) blood may be treated as follows (Bizzozero and Torre, *Archivio per le Scienze mediche*, vol. iv, No. 18, 1880, p. 390): Dilute a drop of blood with 0·75 per cent. salt solution in which has been dissolved a little *methyl violet.* This liquid in no wise affects the form of the elements, stains nuclei intensely, and protoplasm less intensely. May be used for the study of bone-marrow and spleen.

For the staining of the blood-plates of Bizzozero, this observer (*Arch. path. Anat.*; *Zeit. wiss. Mik.*, 1884, p. 389) employs a 0·02 per cent. solution of methyl violet in salt solution, or a 1 : 3000 solution of gentian violet.

Toison (*Journ. Sci. med. de Lille*, fév., 1885; *Zeit. wiss. Mik.*, 1885, p. 398) recommends that blood be mixed with the following fluid:

Distilled water . . .	160 c.c.
Glycerin (neutral, 30° Baumé) .	30 ,,
Pure sulphate of sodium . .	8 grammes.
Pure chloride of sodium . .	1 gramme.
Methyl violet 5 B . .	0·25 ,,

(The methyl violet is to be dissolved in the glycerin with one half of the water added to it; the two salts are to be dissolved in the other half of the water, and the two solutions are to be mixed and filtered.)

Ferrier's liquid is said to have a sp. gr. similar to that of liquor sanguinis. Fuchsin, 1 grm.; water, 150 c.c.; rectified spirit 50 c.c.; dissolve, and add glycerin, 200 c.c. (from Squire's *Methods and Formulæ*, p. 39).

Giglio-Tos (*Zeit. wiss. Mik.*, xv, 1898, p. 166) mixes fresh blood with a saturated solution of neutral red in 0·8 per cent. salt solution, which stains hæmoglobigenous granules in five to ten minutes.

Neutral red is also recommended as an *intra vitam* stain for granules of leucocytes by Ehrlich and Lazarus, see § 318.

Fixed films may be stained with the usual histological stains. Ehrlich's acid hæmatoxylin, § 275, with 0·5 gr. of eosin dissolved in it, is recommended as a general stain.

Ehrlich's triacid, § 307, gives good general views, and demonstrates neutrophilous granules.

Pappenheim's "panoptic triacid" (on sale by Grübler) is Ehrlich's triacid with methylen blue in place of the methyl green.

Ehrlich's mixture for eosinophilous cells has been given (§ 320).

Amongst the host of *methylen blue and eosin* stains may be mentioned the following:

Jenner's mixture (§ 718).

Chenzinsky's mixture (§ 322).

A similar one of Ehrlich ("Anæmie," p. 29): 10 parts of 1 per cent. aqueous eosin, 8 of methylal, and 10 of saturated aqueous methylen blue, fresh mixed.

Michaelis (quoted from *Encycl. mik. Technik*, art. "Blut") makes (*a*) a mixture of 20 parts 1 per cent. aqueous methylen blue with 20 of absolute alcohol, and (*b*), a mixture of 12 parts 1 per cent. aqueous solution of eosin and 28 of acetone, and for staining mixes equal parts of each.

Romanowsky's stain was first published by him in 1891 in a work entitled *Zur Frage d. Parasitologie u. Therapie der Malaria*, and subsequently modified by Ziemann, Zettnow, and others. It appears to differ from the foregoing in that the stain is got by means of Methylenazur (§ 345). Ziemann (*Centralb. Bakteriol.*, xxiv, 1898, p. 945; *Zeit. wiss. Mik.*, xv, 1899, p. 456) stained films for twenty minutes in a mixture of 1 c.c. of 1 per cent. methylen blue (*med. puriss., Höchst*) and 5 c.c. of 1 per cent. eosin solution (mark B.A. or A.G., Höchst). Zettnow (*Zeit. Hygiene*, xxx, 1899, p. 1; *Centralb. Bakteriol.*, xxvii, 1900, p. 803; *Zeit. wiss. Mik.*, xvi, 1899, p. 254 and xvii, 1900, p. 247) added carbonate of soda. 30 c.c. of 1 per cent. sol. of methylen blue (*med., Höchst*) have added to them 3 to 4 c.c. of 5 per cent. solution of carbonate of soda. The mixture may be kept for two or three weeks. For use, to 2 c.c. of the mixture is added with agitation drop by drop 1 c.c. of 1 per cent. sol. of eosin B.A. (Höchst). This mixture is poured on to the cover-glasses, which are allowed to stand for five minutes, rinsed, and differentiated with eosin solution of 1 : 500. According to the *Encycl. mik. Tech.*, p. 87, R. Koch differentiates with distilled water containing a very little acetic acid, until the red of the eosin comes out pure. Red blood corpuscles rose-red, nuclei violet, protoplasm of intraglobular parasites blue, their nuclei bright red.

See also *Centralb. Bakteriol.*, xxx, 1901, p. 248, or *Zeit. wiss. Mik.*, xviii, 1902, p. 314, Reuter's modification; his stain is on sale by Grübler.

Similar in principle is the process of Laveran; see *C. R. Soc. Biol.*, li, 1899, p. 249, or the "Traité" of Lee and Henneguy, 1902, p. 504.

720. Demonstration of Blood-plates of Bizzozero (Kemp, *Studies from the Biol. Lab. Johns Hopkins Univ.*, May, 1886, iii, No. 6; *Nature*, 1886, p. 132).—A somewhat large drop of blood is placed on a slide, and quickly washed with a small stream of normal salt solution. The blood-plates are not washed away, because they have the property of adhering to glass. To make permanent preparations of them, they should first be fixed. This is done by putting a drop of osmic acid solution on the finger before pricking it.

Bizzozero's stain for these elements has been given last §. They may also be stained in films by one of the other methods there given, especially the Romanowsky method. According to Pappenheim ("Farbchemie," p. 107) *Wasserblau* is almost specific for them.

721. Weigert's **Fibrin Stain** (*Fortschr. d. Med.*, v, 1887, No. 8, p. 228; *Zeit. wiss. Mik.*, iv, 1887, p. 512).—Sections (alcohol material) are stained in a saturated solution of gentian or methyl violet in anilin water (§ 298). They are brought on to a slide and mopped up with blotting-paper, and a little Lugol's solution (§ 88) is poured on to them. After this has been allowed to act for a sufficient time they are differentiated and cleared in anilin oil *without previous dehydration with alcohol.* They are simply mopped up with blotting-paper, and a drop of anilin is poured on to them. The anilin soon becomes dark, and is then changed for fresh once or twice. When this has been done, the anilin is *thoroughly* removed by means of xylol, and a drop of balsam and a cover are added. This stain may be applied to celloidin sections without previous removal of the celloidin. See also the modifications of this method by KROMAYER (§ 662) and BENECKE (§ 688).

UNNA (*Monatssch. prakt. Dermat.*, xx, 1895, p. 140; *Zeit. wiss. Mik.*, xiii, 1896, p. 229) gives a modification of the above method, and also one with polychromatic methylen blue and iodide of potassium, and one with fuchsin and tannin.

WOLFF (*Zeit. wiss. Mik.*, xv, 1899, p. 310) makes up the stain with two vols. saturated solution of carbonate of lithia to one of alcoholic gentian or fuchsin; other details *loc. cit.*, or *Journ. Roy. Mic. Soc.*, 1899, p. 234.

722. KOCKEL'S **Fibrin Stain** (*Centralb. allg. Path.*, x, 1899; *Journ. Roy. Mic. Soc.*, 1900, p. 648).—Sections are treated for 5 or 10 minutes with chromic acid of 1 to 5 per cent., washed, and stained for 15 to 20 minutes in WEIGERT'S hæmatoxylin, washed and put into 1 per cent. alum solution until they turn dark blue. They are then washed, differentiated in WEIGERT'S ferricyanide, washed, put for a quarter of an hour into saturated solution of alum, washed and mounted in balsam. They keep well. Fibrin dark blue, keratin and collagen not stained, which is an advantage.

Glands.

723. Mucin.—HOYER (*Arch. mik. Anat.*, xxxvi, 1890, p. 310) finds that the mucin of mucus cells and goblet cells stains with *basic* tar colours and with alum hæmatoxylin, but not with *acid* tar colours. He obtained his best results by means of thionin, and good ones with toluidin blue, both of these giving a metachromatic stain—tissues blue, mucin reddish—and also with methylen blue (which is particularly useful from its power of bringing out the merest traces of mucin), safranin, etc.

All of these colours may be used in the same way. Specimens should be fixed for two to eight hours in 5 per cent. sublimate solution, imbedded in paraffin, cut, and the sections stained for five to fifteen minutes in a very dilute aqueous solution of the dye (two drops of saturated solution to 5 c.c. of water).

Hyaline cartilage, the jelly of Wharton, and the Mastzellen of Ehrlich give the same reactions with basic dyes as mucin does.

These conclusions had already been in part formulated by SUSSDORF (*Deutsche. Zeit. Thiermed.*, xiv, pp. 345, 349; see *Zeit. wiss. Mik.*, vi, 1889, p. 205).

See also the series of papers by BIZZOZERO, "*Sulle ghiandole tubulari del tubo gastro-enterico*," etc., in the *Atti R. Accad. di Sci. di Torino*, 1889 to 1892; reports in *Zeit. wiss. Mik.*, vii, 1890, p. 61; and ix, 1892, p. 219; also UNNA, *ibid.*, xiii, 1896, p. 42.

As regards the safranin reaction, it is well to note that it is not obtained with all brands of the dye; that of Bindschedler and Busch, in Bâle, gives it, whilst safranin 0 of Grübler does not. UNNA employs chiefly polychromatic methylen blue (§ 345).

As regards the thionin stain, see HÁRI, *Arch. mik. Anat.*, lviii, 1901, p. 678.

The subject has been carefully investigated by PAUL MAYER (*Mitth. Zool. Stat. Neapel*, xii, 1896, p. 303. See *last edition*). He gives the following two formulæ for mixtures that stain *exclusively* mucus.

724. MAYER'S **Mucicarmine** (*op. cit.*, last §).—One gramme of carmine is rubbed up in a capsule with 0·5 gramme of aluminium chloride (must be dry, not damp and yellow), and 2 c.c. of distilled water. The capsule is heated over a small flame for two minutes, until the originally light-red mixture has become quite dark. Stir thoroughly. The liquid having become thick, add a little 50 per cent. alcohol, in which the warm mass ought to dissolve easily, and rinse the whole with more alcohol into a bottle. Make up to 100 c.c. with 50 per cent. alcohol, let it stand for at least twenty-four hours, and filter. This gives a stock solution, which is as a rule to be diluted for use tenfold with distilled or tap water. Exceptionally it may be diluted instead five or ten fold with alcohol of 50 per cent. or 70 per cent.

725. MAYER'S **Muchæmatein** (*ibid*).—Hæmatein 0·2 gramme, aluminium chloride 0·1 gramme, glycerin 40 c.c., water 60 c.c.

Rub up the hæmatein in a mortar with a few drops of the glycerin, then add the other ingredients. An alcoholic solution may be made in the same way by dissolving the hæmatein and aluminium chloride in 100 c.c. of 70 per cent. alcohol, with or without the addition of two drops of nitric acid.

726. Mucicarminic Acid (RAWITZ, *Anat. Anz.*, xv, 1899, p. 439).—1 gramme of carminic acid, 2 of aluminium chloride, and 100 c.c. of 50 per cent. alcohol are dissolved and evaporated to dryness on a sand-bath and the residue taken up with 100 c.c. of 50 per cent. alcohol. Use as mucicarmine.

727. Neutral Red.—KULTSCHIZKY (*Arch. mik. Anat.*, xlix, 1897, p. 8) fixes in his mixture (§ 60), and stains sections either in safranin with 2 per cent. acetic acid, or in a similar solution of neutral red (two to three days, washing out with alcohol).

728. Goblet Cells.—So far as these contain mucin they give the reactions above described, see FLEMMING, *Zeit. wiss. Mik.*, 1885, p. 519; PAULSEN, *ibid.*, p. 520; PANETH, *Arch. mik. Anat.*, xxxi, 1888, p. 113 *et. seq.*; and LIST, *ibid.*, xxvii, 1886, p. 481.

RANVIER (*Comptes rend.*, 1887, 3, p. 145) treats the pharyngeal mucosa of the frog first for ten or twelve hours with vapour of osmium, and then for three minutes with vapours of perruthenic acid (RuO_4) and obtains the mucigen in the goblet cells stained black.

729. Salivary Glands.—SOLGER (*Unters. z. Naturlehre d. Menschen*, xv, 5 and 6, pp. 2—15; *Festschr. f. Gegenbaur*, ii, 1896, p. 211) demonstrates the granules in serous cells and half-moons of the submaxillary gland by means of formaldehyde. The gland is hardened for two days or more in a 10 per cent. solution of formol, and may then either be sectioned and examined in the wet way or imbedded in paraffin, and the sections stained with hæmatoxylin of Delafield or of Ehrlich, the granules taking the stain.

KRAUSE (*Arch. mik. Anat.*, xlv, 1895, p. 94) stains sections either with Heidenhain's iron hæmatoxylin or with Ehrlich-Biondi mixture or thionin. See also KRAUSE, *ibid.*, xlix, 1897, p. 709.

730. Gastric Glands.—Kolster (*Zeit. wiss. Mik.*, xii, 1895, p. 314) differentiates the two kinds of cells in stomach glands by over-staining with hæmatoxylin, washing out with alcohol containing 1 per cent. of HCl, blueing with alcohol containing 1 per cent. of ammonia, and, after washing, staining for one to five minutes in a weak solution of Säurefuchsin. Peptic cells blue, parietal cells red. Osmic material cannot be employed.

See also Oppel, *Lehrb. Vergl. mik. Anat. Wirbelthiere*, 1, Jena, 1896.

731. Liver.—Braus (*Denkschr. Med. Nat. Ges. Jena*, v. 1896, p. 307) demonstrates the *bile capillaries* by the rapid method of Golgi, hardening in a mixture of one part formol with three parts liquid of Müller or $\frac{1}{3}$ per cent. chromic acid. He also stains with Bordeaux R and iron hæmatoxylin, or with Ehrlich-Biondi mixture, after fixing in a mixture of one part formol to three of 7½ per cent. sublimate solution.

Eppinger (*Beitr. path. Anat.*, xxxi, 1902, p. 230) studies them by means of a complicated modification of Weigert's neuroglia stain, and Ciechanowski (*Anat. Anz.*, xxi, 1902, p. 426) by means of Weigert's myelin stain (the 1885 method).

Oppel (*Anat. Anz.*, v. 1890, p. 144; vi, 1891, p. 168) puts pieces of liver or spleen (alcohol material) for twenty-four hours into a solution of neutral chromate of potash (½ to 10 per cent.), rinses with a very weak solution of silver nitrate, puts them for twenty-four hours into a ¾ per cent. solution of silver nitrate, washes, dehydrates and cuts without imbedding. The lattice fibres are only stained near the surface, so that tangential sections must be made.

See also Ranvier, *Les membranes muqueuses et le syst. glandulaire,*" *Journ. de Micrograph.*, ix, x, 1885–6; Igacuschi, in *Arch. path. Anat.*, xcvii, p. 142, or *Zeit. wiss. Mik.*, 1885, p. 243 (gold process for study of fibrous networks); Kupffer, *Sitzb. Ges. f. Morph.*, etc., München, Juli, 1889, or *Zeit. wiss. Mik.*, vi, 1889, p. 506 (hæmatoxylin stain for demonstration of ultimate bile-ducts, and application of Golgi's silver bichromate method to the same object and to the study of fibrous networks); Krause (*Arch. mik. Anat.*, xlii, 1893, p. 57).

731a. Spleen.—For *lattice fibres*, see Oppel, last §.

Kultschitzky (*Arch. mik. Anat.*, xlvi, 1895, p. 675) studies the *musculature* in sections (of material from liquid of Müller) stained for a day or more in a solution of "lakmoid" in ether and mounted in balsam.

For *elastic fibres* he puts sections for half an hour or a day into a mixture of 800 parts 96 per cent. alcohol, 40 parts 1 per cent. solution of carbonate of potash, 2 parts Magdala red, and 1 part methylen blue.

For the *blood-vessels* he puts sections of Müller material for a few minutes into a solution of one or two parts of Säurerubin in 400 parts of 3 per cent. acetic acid, washes out in 2 per cent. acetic acid, and after-stains in a similar solution of helianthin or Wasserblau until the red only remains in the erythrocytes.

See also WHITING (*Trans. Roy. Soc.*, Edinburgh), xxxviii, 1896, p. 311.

732. Kidney.—SAUER (*Arch. mik. Anat.*, xlvi, 1895, p. 110) finds that for the renal epithelium the best fixative is Carnoy's acetic alcohol with chloroform, § 90 (three to five hours, washing out with absolute alcohol). A mixture of nine parts alcohol with one of nitric acid is also good, as is liquid of Perényi. He stains with iron hæmatoxylin, and after-stains in a very weak solution of Säurerubin in 90 per cent. alcohol, which stains the ciliary plateau. He macerates with iodised serum or one third alcohol, staining afterwards with dahlia.

ARNOLD (*Anat. Anz.*, xxi, 1902, p. 417) employs *intra vitam* staining methods (§ 221) for the study of the granules of the epithelium cells. Sections of fresh kidney are cut with a Valentin's knife, and brought into a very dilute solution of neutral red, or methylen blue, in which the granules stain in a few minutes or hours. Or saturated solutions of the dyes, or of indigo carmine, may be injected subcutaneously during life, at intervals of fifteen to twenty minutes, and after two to five injections the organ may be excised and sections made and examined (see §§ 221 and 350 to 352).

CHAPTER XXXI.

NERVOUS SYSTEM—GENERAL METHODS.

733. Introduction.—Microscopical research into the structure of the nervous system pursues two ends. Either it is desired to elucidate the minute structure of the nervous elements or neurons—that is to say, the internal organisation of nerve-cells and nerve-fibres, the processes employed to this end forming a group of *cytological* methods; or it is desired to study the form of nerve-cells, the exact distribution of the divers groups of nerve-cells in the grey matter, the connections that are formed by means of nerve-fibres between these groups of nerve-cells or "nuclei," and to follow out the intricate course of the tracts of fibres that enter into the constitution of the white matter of the cerebrospinal axis. The processes employed in all these researches form a group of the *anatomical* methods of neurology.

This chapter is devoted to the special section methods employed for the *central* nervous system, and to some methods of staining which are useful for general views of nervous organs, thus forming an introduction to the anatomical methods. The cytological methods will be given in the next chapter, the special anatomical methods for medullated nerve tracts (the WEIGERT group) in Chap. XXXIII, those for non-medullated tracts and cell-processes (the GOLGI group) in Chap. XXXIV, and the methods for neuroglia and some nerve end-organs in Chap. XXXV. But as many of these methods overlap, strict classification is not always possible, and the reader who may not find that which he requires under one head is requested to look for it under the others.

A large proportion of the methods used in the study of nerve-tissue in *peripheral* organs having already been extensively treated of in the chapters on "Methylen Blue," on "Impregnation Methods," on "Tegumentary Organs," and on "Muscle and Tendon," those chapters should be referred to in order to complete the account given in the following pages.

For more minute details concerning the dissection and hardening of the voluminous encephala of Man and the larger Vertebrates than can be given here see MERCIER, *Les Coupes du Système Nerveux Central* (1894, Paris, Rueff); DÉJÉRINE, *Anatomie des Centres Nerveux*, Paris, 1895; BEVAN LEWIS, *The Human Brain; Histological and Coarse Methods of Research*, London, Churchill; OBERSTEINER, *Anleitung beim Studium des Baues d. nervösen Centralorgane im gesunden u. kranken Zustande*, Leipzig, Toeplitz; and VAN WALSEM, *Verh. Akad. Wetensck. Amsterdam*, vii, 1899.

SECTION METHODS.

734. Fixation by Injection.—Fixation, in the proper sense of the word, is, of course, out of the question in the case of the human subject. But in the case of the lower animals it is possible to introduce fixing liquids into the living nerve-centres by means of injection, thus ensuring a much more rapid penetration of the reagents than can be obtained by simple immersion. This method was, I believe, first suggested by GOLGI (*Arch. Ital. de Biologie*, t. vii, 1886, p. 30). He injected 2·5 per cent. solution of bichromate of potash through the carotid if he wished to limit the hardening to the encephalon, or through the aorta if he desired to fix the spinal cord.

DE QUERVAIN (*Virchow's Archiv*, cxxxiii, 1893, p. 489) proceeds in a similar manner, injecting solution of Müller warmed to body heat. For dogs 300 to 400 c.c. are required, for cats one third to one half that quantity. After injection the whole organ is put into solution of Müller for some weeks. (Further details in *fourth edition.*)

MANN (*Zeit. wiss. Mik.*, xi, 1894, p. 482) injects through the aorta. Before throwing in the fixing liquid, he injects for about twenty seconds physiological salt solution warmed to 39° C. This washes out the capillaries, and prevents the blood from coagulating there. The fixing solution employed by him consists of saturated solution of corrosive sublimate, warmed to 39° C. After five minutes of injection the brain ought to be fixed throughout. It is removed and put for twelve hours into the same sublimate solution, after which it is either put for permanent preservation into 0·1 per cent. solution of sublimate, or is at once passed through alcohol for imbedding in paraffin.

See also § 745 (GEROTA) and STRONG (*New York Acad. of Sci.*, January 13th, 1896; *Anat. Anz.*, xi, 21, 1896, p. 655; *Journ. Comp. Neurol.*, xiii, 1903, p. 291); and MCFARLAND (*Journ. App. Micr.*, ii, 1899, p. 541).

HARDENING.

735. Hardening by the Freezing Method.—The ether freezing method is to be preferred. The sections should be floated on to water, treated for a minute on the slide with 0·25 per cent. osmic acid solution, and stained or otherwise treated as desired. See §§ 195 to 197.

For a detailed description of these manipulations see BEVAN LEWIS'S *The Human Brain*.

736. Hardening by Reagents.—If large pieces of nerve-tissue are to be hardened, it is necessary to take special precautions in order to prevent them from becoming deformed by their own weight during the process. Spinal cord or small specimens of any region of the encephalon may be cut into slices of a few millimetres' thickness, laid out on cotton-wool, and brought on the wool into a vessel in which they may have the hardening liquid poured over them. The wool forms an elastic cushion on which the preparations may lie without being distorted by their own weight, and allows the reagent to penetrate by the lower surfaces of the preparations as well as by their exposed surfaces. Or, still better, the preparations should be *suspended* in the liquid, see § 32.

Another plan which I used to follow some five-and-twenty years ago, and which I have seen lately recommended (*Encycl. mik. Technik.*, p. 958) is to add to the hardening liquid enough glycerin to make the preparations *just* float.

If the preparations are placed on the bottom of the vessel, they should never be placed one on another.

If it be desired to harden voluminous organs without dividing them into portions, they should at least be incised as deeply as possible in the less important regions. It is perhaps better in general not to remove the membranes at first (except the dura mater), as they serve to give support to the tissues. The pia mater and arachnoid may be removed partially or entirely later on, when the hardening has already made some progress.

The *spinal cord,* the *medulla oblongata,* and the *pons Varolii* may be hardened *in toto.* The dura mater should be removed at once, and the preparation hung up in a cylinder-glass with a weight attached to its lower end. The weight serves to prevent the torsions of the tissues that may otherwise be brought about by the elastic fibres of the pia mater and arachnoid.

The *cerebrum* should have plugs of cotton-wool put into the fissure of Sylvius, and as far as possible between the convolutions. Unless there are special reasons to the contrary, the brain should be divided into two symmetrical halves by a sagittal cut passing through the median plane of the corpus callosum. BETZ recommends that after a few hours in the hardening liquid the pia mater should be removed wherever it is accessible, and the choroid plexuses also. I have found this by no means easy.

The cerebellum should be treated after the same manner.

The temperature at which the preparations are kept in the hardening solution is an important point. The hardening action of most solutions is greatly enhanced by heat. Thus WEIGERT (*Centralb. med. Wiss.*, 1882, p. 819; *Zeit. wiss. Mik.*, 1884, p. 388) finds that at a temperature of from 30° to 40° C. preparations may be sufficiently hardened in solution of Müller in eight or ten days, and in solution of Erlicki in four days, whilst at the normal temperature two or three times as long would be required.

But it is not certain that this rapid hardening aways gives the best results. SAHLI, who has made a detailed study of the hardening action of chrome salts, is of opinion that it does not (see *Zeit. wiss. Mik.*, 1885, p. 3), and other authors are of the same opinion.

On the other hand, the slowness of the action of chromic salts at the normal temperature is such that decomposition may easily be set up in the tissues before the hardening and preserving fluid has had time to do its work. For this reason voluminous preparations that are to be hardened in the slow way should be put away in a very cool place—best of all in an ice-safe. A hemisphere will require eight or nine months for hardening in this way.

See also PFISTER in *Neurol. Centralb.*, xvii, 1898, p. 643 (*Zeit. wiss. Mik.*, xv, 1899, p. 494).

737. The Reagents to be employed.—The hardening agents most used are the *chromic salts.* Chromic acid was much used at one time, but most workers now agree that its action, though much more rapid than that of the salts, is much more uneven, and frequently causes a disastrous friability of the tissues. Osmic acid can hardly be used for objects of more than a cubic centimetre in size at most.

The liquid of ERLICKI has a more rapid action than the other solutions of chromic salts. SAHLI, however (*loc. cit.*, last §), after having studied the action of the usual solutions, concludes that the best hardening agent for fresh tissues is *pure bichromate of potash,* in 3 or 4 per cent. solution, the hardening being done in a cold place. He rejects the liquid of Erlicki on account of the precipitates it so frequently gives rise to (see § 58).

OBERSTEINER is of the same opinion, recommending pure bichromate for general hardening purposes; whilst for the study of the most delicate structural relations he recommends fixing in Fol's modification of Flemming's liquid (§ 46) for twenty-four hours, followed by washing with water and hardening in 80 per cent. alcohol.

In view of the slowness of penetration of chromic salts, it is often advisable to treat preparations for twenty-four hours or more with alcohol of 80 to 90 per cent. before putting them into the hardening liquid, in order to avoid maceration of the deeper layers of tissue.

BURCHARDT (*La Cellule*, xii, 1897, p. 337) says that "according to the unanimous judgment of all investigators the bichromates of potash and ammonia should not be employed for the *cytological* study of nerve-cells." NISSL (*Encycl. mik. Technik*, p. 969) holds that, for this purpose, only alcohol, formol, sublimate, and occasionally nitric acid, are admissible.

Several observers have lately been using *acetic alcohol.* So TIMOFEEW, *Intern. Monatsschr. Anat. u. Phys.*, xv, 1898, p. 259 (CARNOY's second formula, § 90).

Ohlmacher recommends his sublimate mixture, § 72.

For formaldehyde see §§ 115, 120, 745, and 819.

Chromic acid is not much used alone (see § 41). A very little (say one to two drops of 1 per cent. solution for each ounce) added to bichromate solution will do no harm, and will quicken the hardening.

Nitric acid has been, and still is, employed in strengths of 10 to 12 per cent., and gives particularly tough preparations.

Neutral acetate of lead in 10 per cent. solution affords an excellent preservation of ganglion cells, according to Anna Kotlarewski (see *Zeit. wiss. Mik.*, iv, 1887, p. 387).

Trzebinski (*Virchow's Arch.*, 1887, p. 1; *Zeit. wiss. Mik.*, iv, 1887, p. 497) finds that, as regards the faithful preservation of ganglion cells (of the spinal cord of the rabbit and dog), the best results are obtained by hardening for eight days in 7 per cent. solution of *corrosive sublimate*, followed by hardening in alcohol containing 0·5 per cent. of iodine. Similarly, Diomidoff (*ibid.*, p. 499), with brain. This process produces artificial "pigment spots," similar to those produced by solution of Erlicki; they may be dissolved out by prolonged treatment with warm water, or in five minutes by strong solution of Lugol. The tissues are of a good consistence for cutting.

Fish (*The Wilder Quarter-Century Book*, 1893, p. 335) and Donaldson (*Journ. of Morphol.*, ix, 1894, p. 123) have made determinations of weight and volume, with the object of ascertaining what changes are produced by reagents in encephala of sheep. They have found that bichromate of potash produces a slight increase both in weight and volume, whereas all the other reagents tried produce a diminution of both these factors.

Flatau (*Anat. Anz.*, xiii, 1897, p. 323) finds that brain augments in weight slightly in 10 per cent. formol solution (spinal cord somewhat more); whilst in 1 per cent. solution it may increase as much as 24 per cent.

738. Strengths of the Reagents.—All hardening reagents (except osmic acid) should at first be taken as weak as is consistent with the preservation of the tissue, and be changed by degrees for stronger.

Osmic acid may be taken of 1 per cent. strength, and will harden small pieces of tissue sufficiently in five to ten days (Exner).

Bichromate of potash should be taken at first of not more that 2 per cent. strength; this is then gradually raised to 3 or 4 per cent. for the cord and cerebrum, and as much as 5 per cent. for the cerebellum. Obersteiner begins with 1 per cent., and proceeds gradually during six to eight weeks to 2 or 3 per cent. (This is at the normal temperature;

at a temperature of 35° to 45° C. the hardening can be got through in one or two weeks).

Bichromate of ammonia should be taken of half the strength recommended for bichromate of potash, or even weaker at first; it may be raised to as much as 5 per cent. for cerebellum towards the end of the hardening.

739. Betz's **Methods** (*Arch. mik. Anat.*, 1873, p. 101).—Brain and spinal cord are first hardened, for some days or weeks, in 70 to 80 per cent. alcohol containing enough tincture of *iodine* to give it a light brown coloration. (As fast as the alcohol becomes colourless by absorption of the iodine by the tissues more iodine must be added.) After this they are definitely hardened in bichromate of potash of 3 per cent. for spinal cord, medulla oblongata, and pons, 5 per cent. for cerebellum, and 4 per cent. for cerebrum.

For details see *early editions*.

740. Cerebrum. (Bevan Lewis, *The Human Brain*, p. 102).—Methylated spirit, twenty-four hours in a cool place. Müller's solution, three days in a cool place. Then change the liquid; and after three days more change it again, or, preferably, substitute a 2 per cent. solution of potassium bichromate. At the end of the second week a solution of double the strength may be added; and if at the termination of the third week the mass is still pliable, and of the consistence of ordinary rubber, it is as yet unfit for section-cutting, and the reagent should be replaced by a solution of chromic acid.

741. Brain (Hamilton, *Journ. of Anat. and Physiol.*, 1878, p. 254). Put into—

Müller's fluid	3 parts.
Methylated spirit	1 part.

Put away in an ice-safe. Change the solution in a fortnight or three weeks; or if on examining a section of one of the pieces it is found that the hardening reagent has penetrated to the interior, they may be at once removed to the following mixture:

Bichromate of ammonia . . .	1 grm.
Water	400 c.c.,

in which they remain for one week. Then change the solution to one of 1 per cent. for one week, and let this be followed by a solution of 2 per cent. for another week, or longer if required. The pieces will now be sufficiently hard for cutting; they may be kept permanently in solution of chloral hydrate, twelve grains to the ounce.

742. Entire Encephalon (DEECKE, *Journ. Roy. Mic. Soc.*, 1883, p. 449).—Bichromate of ammonia in $\frac{1}{2}$ to 1 per cent. solution, according to the consistence of the brain. If it is soft he adds, say, $\frac{1}{6}$ to $\frac{1}{10}$ per cent. of chromic acid to the solution, and always $\frac{1}{6}$ to $\frac{1}{4}$ of the whole volume of alcohol.

Further details in *early editions*.

743. Encephalon (M. DUVÁL, ROBIN'S *Journal de l'Anatomie*, 1876, p. 497).—Bichromate of potash 25, water 1000; change the liquid after the first twenty-four hours, and again after three or four days. After two or three weeks place the preparations in chromic acid of 3 per 1000 (with a few lumps of camphor to prevent mould), change the liquid every day for the first week and after that every eight days until the middle of the second month. The preparations must remain at least two months in the chromic acid.

744. Encephalon (FISH, *The Wilder Quarter-Century Book*, 1893, p. 393).

Water	400 c.c.
95 per cent. alcohol	400 ,,
Glycerin	250 ,,
Zinc Chloride	20 grms.
Sodium Chloride	20 ,,

Immerse in this for about three days, then transfer for a week or more to a mixture of equal parts of the fluid and 70 per cent. alcohol, and finally store in 90 per cent. alcohol.

745. Formaldehyde.—*For formaldehyde in general* see § 115.

WEIGERT (*Beit. Kenntn. norm. mensch. Neuroglia*, 1895, p. 1146) puts portions of material of not more than half a centimetre in thickness for four days into a "4 per cent. solution of formol."

MARCUS (quoted from FISH, see below) hardens spinal cord for two or four weeks in a ½ per cent. solution of formalin, then small pieces one half-centimetre thick are cut out and placed in Müller's fluid for a week in an oven at 37° C.

Van Gieson (*Anat. Anz.*, x, 1895, p. 494) has used "solutions of formalin of 4, 6, and 10 per cent.," followed by 95 per cent. alcohol. Myelin was found to be well preserved and to give the characteristic blue reaction with Weigert's hæmatoxylin (the 1885 method), just as if a chrome salt were present.

Lachi (*cf. Zeit. wiss. Mik.*, xii, 1895, p. 32) has had good results with "20 per cent. solutions of formol."

Fish (*Proc. Amer. Mic. Soc.*, xvii, 1895, p. 319) recommends:

Water	2000	c.c.
Commercial formalin	50	,,
Sodium chloride	100	grms.
Zinc chloride	15	,,

Brains should be left in this mixture for a week or ten days or more, then transferred to a solution of water 2000 c.c., formalin 50 c.c., in which they may remain indefinitely if the jar be kept tightly covered.

Parker and Floyd (*Anat. Anzeiger*, Bd. xi, 1895, p. 156) advise (for sheep's brains) a mixture of—

Alcohol 95 per cent.	6	volumes
Formol 2 per cent.	4	,,

Brains may be kept for months in the mixture (*ibid.*, 1896, p. 568).

Gerota (*Zeit. wiss. Mik.*, xiii, 1896, p. 314) puts human brains into a 5 or 10 per cent. solution of formol, and after twenty-four hours removes the pia and changes the liquid; this is also further done every five to seven days, and in one or two weeks the hardening is complete. In the case of fœtal brains of *Canis*, *Felis*, and *Homo*, he first injects the vascular system with a 10 to 15 per cent. solution of formol in 85 per cent. alcohol, and then brings the heads into the 5 to 10 per cent. watery solution; after one or two days he removes the brains from the skull and puts them back for fifteen to twenty days into the formol.

Orth (*Berl. klin. Wochenschr.*, 1896, No. 13; *Zeit. wiss. Mik.* xiii, 1896, p. 316) recommends *Formol-Müller* § 120. It must be changed every few days. Small pieces of tissue may be sufficiently hardened in a few hours in a stove. This mixture is now very popular.

Nelis (*Bull. Acad. Sc. Belg.*, 1899, 1900, p. 726) fixes spinal ganglia for twenty-four hours in a solution of 20 gr. sulphate of copper, and sublimate to saturation, in a litre of 7 per cent. formol with 5 c.c. of acetic acid.

For special mixtures for Golgi impregnations see § 819.

746. Nervous Centres of Reptiles, Fishes, and Amphibia.—Mason (*Central Nervous System of Certain Reptiles*, etc.; Whitman's *Methods*, p. 196) recommends iodised alcohol, six to twelve hours; 3 per cent. bichromate, with a piece of camphor in the bottle, and to be changed once a fortnight until the hardening is sufficient (six to ten weeks).

Burckhardt (*Das Centralnervensystem von Protopterus*, Berlin, 1892; *Zeit. wiss. Mik.*, ix, 1893, p. 347) recommends a liquid composed of 300 parts of 1 per cent. chromic acid, 10 parts of 2 per cent. osmic acid, and 10 parts of concentrated nitric acid, in which brains of Protopterus are hardened in twenty-four to forty-eight hours.

Fish (*Journ. of Morphol.*, x, 1, 1895, p. 234) employed for the encephalon of *Desmognathus fusca* a mixture of 100 c.c. of 50 per cent. alcohol, 5 c.c. of glacial acetic acid, 5 grms. of corrosive sublimate, and 1 grm. of picric acid, fixing for twelve to twenty-four hours, and passing through the usual alcohols.

747. Giacomini's **"Dry" Process for Preserving Brains** (*Arch. per le Scienze Mediche*, 1878, p. 11). See *early editions*.

Imbedding and Cutting.

748. The Methods of Imbedding.—The paraffin infiltration method can only conveniently be used for the smaller objects of this class. Human spinal cord can be properly infiltrated with paraffin by first cutting it up into slices of not more than a few millimetres thick. The largest objects of this class, such as entire hemispheres of man, cannot be *really infiltrated* with any known imbedding mass in any reasonable time: and the anatomist must be content with simple superficial imbedding—the mere production of a mould of imbedding mass round the tissues. For intermediate objects—those whose size varies between that of a small nut and a walnut—it appears to me that they are best treated by the collodion method, which is at once the safest, the most convenient, and the most advantageous as regards the ulterior treatment of sections.

Imbedding is not a necessary process. Sections can be obtained from any part of the central nervous system without imbedding. The material should be well hardened, and a suitable piece should be glued on to a piece of wood or cork by means of a rather thick solution of gum arabic. As soon as it begins to stick to the support the whole is thrown into 80 per cent. alcohol to harden the joint, after which it may be fixed in the object-holder of the microtome and cut.

If the collodion method has been taken it may be found that, notwithstanding every precaution, the collodion has not thoroughly penetrated the tissues. Good sections may, however, still be obtained by DUVAL's method of collodionising the sections. The cut surface of the tissue is dried by blowing on it, and is covered with a thin layer of collodion laid on it with a brush. As soon as this layer has somewhat dried, which happens very rapidly, a section is cut and the cut surface is collodionised as before, and so on for each section. This process gives very good results, and may be advantageously employed even with material that has been successfully imbedded, as it gives a better consistency to the tissue, and enables thinner sections to be obtained (VAN GEHUCHTEN, *in litt.*).

STRASSER (*Zeit. wiss. Mik.*, ix, 1892, p. 8) obtains paraffin sections of 10 cm. breadth by 15 cm. length. He cuts out from hardened material slices of from 1 to 2 cm. in thickness, de-alcoholises them with xylol-carbolic acid mixture, § 179, allows this to evaporate, and brings them first into melted yellow vaselin, and lastly either into a mixture of vaselin and paraffin of 42° melting point, or into pure paraffin.

He also imbeds the slices in celloidin, and clears them before cutting with a mixture of xylol-carbolic acid and 80 per cent. alcohol in equal parts.

FEIST (*Zeit. wiss. Mik.*, viii, 1892, p. 492) marks the right and left sides of spinal cord by imbedding with each segment of it a small cylinder (of about 1 square millimetre in section) of hardened liver, stuck vertically in the imbedding mass (either celloidin or paraffin) against the side of the cord that it is desired to mark.

For the *freezing method* see p. 136, and for further details concerning imbedding and cutting see *fourth edition.*

GENERAL STAINS.

749. *Ammonia-carmine* may be used for general views. The secret of success lies in staining very slowly in extremely dilute solutions. Bichromate material ought to be brought *direct into the stain* without passing through alcohol (see § 55).

Picro-carmine has much the same action as ammonia-carmine, but gives a better demonstration of non-nervous elements.

Chromic objects stain very slowly in both these media. Sections may, however, be stained with them in a few minutes if they be put into a watch-glass with the stain, and the whole be kept on a wire net over a water-bath heated to boiling-point (OBERSTEINER).

HENLE (*Handb. d. Nervenlehre,* 1871) gives the following, after MERKEL. Sections should be placed in solution of chloride of palladium (1 in 300 to 1 in 600) till they are of a straw-colour (one or two minutes), rinsed in water, and stained in strong ammonia-carmine. Myelin, yellow; axis-cylinders, nerve-cells, and neuroglia, deep red.

Borax-carmine is useful when employed for double-staining with indigo-carmine or an anilin blue to follow. I have obtained some useful stains with Seiler's borax-carmine and indigo-carmine process (§ 393).

Recent authors recommend *soda-carmine.* The *Encycl. mik. Technik,* p. 927, advises staining Müller material for a couple of days in a 2 per cent. solution of carminate of soda (Grübler's).

See also SCHMAUS (*Münch. med. Wochenschr.*, 1891, No. 8; *Zeit. wiss. Mik.*, viii, 1891, p. 230); UPSON (*Neurolog. Centralb.*, 1888, p. 319; *Zeit. wiss. Mik.*, v, 1888, p. 525); FREEBORN (*Amer. Mon. Mic. Journ.*, 1888, p. 231; *Journ. Roy. Mic. Soc.*, 1889, p. 305); KADYI, *Neurol. Centralb.*, xx, 1901, p. 687; *Zeit. wiss. Mik.*, xviii, 1902, p. 483); CHILESOTTI (*ibid.* xix, 1902, p. 161, and xx, 1903, p. 87).

750. Anilin blue-black has been much recommended by SANKEY (*Quart. Journ. Mic. Sci.*, 1876, p. 69); BEVAN LEWIS (*Human Brain*, p. 125; VEJAS (*Arch. f. Psychiatrie*, xvi, p. 200); GIERKE (*Zeit. wiss. Mik.*, 1884, p. 376); MARTINOTTI (*ibid.*, p. 478); JELGERSMA (*Zeit. wiss. Mik.*, 1886, p. 39); SCHMAUS (*Münch. med. Wochenschr.*, No. 8, 1891, p. 147; *Zeit. wiss. Mik.*, viii, 1891, p. 230), and others. As to this colour see § 336, and for details see *previous editions.*

751. Picronigrosin.—MARTINOTTI (*loc. cit.*, 1884, p. 478) stains for two or three hours or days in a saturated solution of nigrosin in saturated solution of picric acid in alcohol, and washes out in a mixture of 1 part of formic acid with 2 parts of alcohol until the grey matter appears clearly differentiated from the white to the naked eye.

752. KAISER (*Zeit. wiss. Mik.*, vi, 1889, p. 471) stains sections of spinal cord for a few hours in a solution of 1 part of naphthylamin brown, 200 of water, and 100 of alcohol, washes with alcohol, clears with origanum oil, and mounts.

753. Alizarin.—SCHRÖTTER (*Neurol. Centralb.*, xxi, 1902, p. 338; *Zeit. wiss. Mik.*, xix, 1903, p. 381) stains sections for twenty-four hours in a 1 to 2 per cent. solution of sulphalizarinate of soda, differentiates for $\frac{1}{2}$ to 1 minute in tap-water, dehydrates, and mounts. This is a general stain, but demonstrates Nissl bodies and other internal details.

754. WOLTER'S **Chloride of Vanadium** process (*Zeit. wiss. Mik.*, vii, 1891, p. 471):

The material (either central or peripheral nervous tissue) is *hardened in the bichromate liquid of* KULTSCHITZKY, § 59, followed by alcohol, *as there described*. Sections are mordanted for twenty-four hours in a mixture of 2 parts of 10 per cent. solution of chloride of vanadium and 3 parts of 3 per cent. solution of acetate of aluminium, washed for ten minutes in water, and stained for twenty-four hours in a solution of 2 grammes of hæmatoxylin (dissolved in a little alcohol) in 100 c.c. of 2 per cent. acetic acid. They are washed out until they are of a light blue-red colour in 80 per cent. alcohol acidulated with 0·5 per cent. of hydrochloric acid. Remove the acid thoroughly by washing with pure alcohol, dehydrate, clear with origanum oil, and mount.

Chiefly an axis-cylinder stain, myelin being coloured only if the differentiation in the acid alcohol is insufficient, but cells are also stained.

755. SCARPATETTI (*Neurol Centralb.*, xvi, 1897, p. 211; *Zeit. wiss. Mik.*, xiv, 1897, p. 91) obtains an axis-cylinder and cell-stain as follows:—Sections of formol material are stained for five minutes in 1 per cent. hæmatoxylin, treated for five minutes with concentrated solution of neutral acetate of copper, differentiated with Weigert's borax-ferricyanide, then treated with concentrated solution of carbonate of lithia, washed, and mounted. Myelin is not stained.

756. **Chrom-Hæmatoxylin.**—For the troublesome method of FAJERSTAJN (*Poln. Arch. Biol.* 1901, p. 3) see *Zeit. wiss. Mik.* xviii, 1902, p. 479.

757. MALLORY's **Phospho-molybdic-acid Hæmatoxylin** has been given, § 286.

For the extremely complicated modification of AUERBACH, see *Neurol. Centralb.*, xvi, 1897, p. 439, or *Zeit. wiss. Mik.*, xiv, 1897, p. 402, and for that of KODIS see § 286.

758. **Hæmatoxylin and Säurefuchsin.**—FINOTTI (*Virchow's Arch.*, cxliii, 1896, p. 133; *Zeit. wiss. Mik.*, xiii, 1896, p. 236) stains in hæmatoxylin, washes out well, counter-stains for three minutes with 0·5 to 1 per cent. solution of Säurefuchsin, and differentiates in 75 per cent. alcohol containing a very little caustic potash.

VAN GIESON's hæmatoxylin and picro-Säurefuchsin, § 408, gives useful general views of nerve-cells, axis-cylinders, and neuroglia.

759. ALT (*Münch. med. Wochenschr.*, 1892, No. 4; *Zeit. wiss. Mik.*, ix, 1, 1892, p. 81) stains for a couple of hours in solution of Congo in absolute alcohol, and washes out with pure alcohol. For peripheral axis-cylinders, and other elements.

CHAPTER XXXII.

NERVOUS SYSTEM—CYTOLOGICAL METHODS.

760. Introduction.—In this chapter are grouped together methods for the study of the minute structure of nerve-cells, of axis-cylinders, and of their medullary sheath.

The ordinary methods of cytology are of course available for nerve-cells. But there are two characteristic elements of these cells—the tigroid substance, and the system of neurofibrils, which require, for minute study, special methods such as the following.

A. *Cells.*

761. Tigroid substance is a markedly basophilous element, occurring in the form of granules or larger irregular blocks known as the "bodies of Nissl." It takes up basic anilin dyes, but does not hold them with such special energy, as for example, the chromatin of nuclei. It is usually stained by the regressive method, with very careful differentiation. The material is usually fixed with alcohol, formol, or sublimate. Van Gehuchten and Nelis (*La Cellule*, xiv, 1898, p. 374) much recommend Gilson's mixture, § 74. Either the method of staining of Nissl may be followed, or one of the others given, according to circumstances. The lower vertebrates are not good subjects.

762. Tigroid Substance,—Nissl's **Methylen-blue Method** (*Neurol. Centralb.*, 1894, p. 508).—Fresh material is hardened in 96 *per cent. alcohol*, and sectioned *without imbedding*. The sections are *floated* on to the following stain poured into a watch-glass :

Methylen blue (Methylenblau B. pat.) .	3·75	parts.
Venice soap	1·75	„
Distilled water	1000·0	„

(This stain is best not used fresh, but kept for some months.)

The watch-glass is warmed over a flame to about 65° to 70° C., till bubbles are given off which burst at the surface of the liquid. The sections are then brought for an instant (5 to 20 seconds) into a mixture of 10 parts of anilin oil with 90 parts of 96 per cent. alcohol, and as soon as no more colour is given off from them are got on to a slide, dried with filter-paper, cleared with oil of cajeput, dried again with filter-paper, treated with a few drops of benzin, and mounted in benzin-colophonium, or (*op. cit.*, *infra*) xylol-colophonium.

See also *op. cit.*, p. 781, and the description in *Encycl. mik. Technik*, pp. 992–997. The process of igniting the benzine (*last ed.*) seems now abandoned.

Prof. VAN GEHUCHTEN writes me that he prefers to take *paraffin sections*, mounted on slides by the water method (§ 200), and stain them for five or six hours in Nissl's mixture in a stove kept at 35° to 40° C. Differentiation is done as above, the cajeput oil is removed with xylol, and the sections are mounted in xylol-damar.

REHM (*Münch. med. Wochenschr.*, 1892, No. 13; *Zeit. wiss. Mik.*, ix, 1893, p. 387), stains for half a minute to a minute in a *hot* 0·1 per cent. of methylen blue, washes in 96 per cent. alcohol till no more colour comes away, clears with origanum oil, and mounts in balsam.

GOTHARD (*C. R. Soc. Biol.*, v, 1898, p. 530) stains celloidin sections for twenty-four hours, *without heat*, in Unna's polychromatic methylen blue and differentiates in a mixture of 5 parts of creosote, 4 of oil of cajeput, 5 of xylol, and 16 of absolute alcohol. The sections should be first dipped in alcohol, and then have two or three baths in the mixture, the whole taking 15 to 20 minutes (*Sem. Med.*, 1900, p. 51).

LUITHLEN and SORGO (*Neurol. Centralb.*, xvii, 1898, p. 640; *Zeit. wiss Mik.*, xv, 1899, p. 359) differentiate in Unna's glycerin-ether mixture (§ 703), remove this with absolute alcohol, and clear in origanum oil.

EWING (*New York Med. Record*, 1898, p. 513; *Zeit. wiss Mik.*, xvi, 1899, p. 95) prefers to differentiate simply in absolute alcohol, and clear in oil of cajeput.

See also GOLDSCHEIDER & FLATAU, *Normale und. path. Anat. der Nervenzellen*, etc., Berlin, Kornfeld, 1898 (*Zeit. wiss. Mik.*, xvi, 1899, p. 102), and NISSL's remarks thereon, *Deutsche Zeit. Nervenheilk.*, xiii, 1899, p. 348 (*Zeit. wiss. Mik.* xvi, 1899, p. 370).

Further, Cox *Intern. Monatsschr. Anat. Phys.*, xv, 1898, Heft. 8; *Zeit. wiss. Mik.*, xvi, 1899, p. 101.

763. Tigroid Substance,—Methylen Blue and Erythrosin.—Held (*Arch. Anat. Phys., Anat. Abth.*, 1895, 1896, p. 399) stains sections on slides, with the aid of a gentle heat, for one or two minutes in a solution of 1 grm. of Grübler's erythrosin in 150 of water with two drops of glacial acetic acid, washes out with water, and stains in a mixture of equal parts of Nissl's methylen blue and 5 per cent. solution of acetone, warming strongly the while, until all odour of acetone has disappeared. After cooling he differentiates with 0·1 per cent. solution of alum until the sections appear reddish, rinses in water, dehydrates as rapidly as possible in absolute alcohol, and passes through xylol into balsam. See further hereon Held, *op. cit.*, 1897, pp. 226—233, 273—305 (Supplementband), and Boccardi, *Mon. Zool. Ital.*, x, 1899, p. 141; *Zeit. wiss Mik.*, xvi, 1900, p. 471 (stains in a mixture of erythrosin 0·1, toluidin blue, 0·2, and water 100 parts, and differentiates in 0·5 per cent. alum solution).

764. Tigroid Substance,—Thionin. Lenhossék (*Fein. Bau. d. Nervensystems*, Berlin, 1894, p. 149) stains sections of *formol material* for five minutes in a concentrated aqueous solution of thionin, rinses with water, differentiates in a mixture of 1 part anilin oil to 9 of absolute alcohol, and passes through oil of cajeput or xylol into damar or balsam. The stain does not keep well.

Similarly Ramón y Cajal, *Man. de Anat. Path. Gen.*, 1896 (see *Zeit. wiss. Mik.*, xv, 1899, p. 375), and Luxenburg, *Neurol. Centralb.*, xviii, 1899, p. 629; *Zeit. wiss. Mik.*, xvi, 1900, p. 477.

765. Tigroid Substance,—Toluidin Blue.—Lenhossék (*Neurol. Centralb.*, xvii, 1898, p. 577; *Zeit. wiss. Mik.*, xv, 1899, p. 492). Sections are stained on slides for a night in concentrated solution of toluidin blue, rinsed in water, quickly differentiated with alcohol, cleared with xylol or carbolic-acid xylol, and mounted in balsam. They may be very lightly counter-stained with erythrosin before the differentiation.

Similarly Polumordwinow (*Zeit. wiss. Mik.*, xvi, 1899, p. 371), who stains in a very weak alkaline solution, 1 part of 1 per cent. solution to 119 of water and 1 of carbonate of soda.

766. Tigroid Substance,—Neutral Red.—Juliusburger (*Neurol. Centralb.*, xvi, 1897, p. 259; *Zeit. wiss. Mik.*, xiv, 1897, p. 211) stains sections of formol material for half to three quarters of a minute in *warm* 1 per cent. solution of neutral red, dehydrates in alcohol, and passes through bergamot oil to balsam.

Rosin (*Deutsche med. Wochenschr.*, 1898, No. 39, p. 615; *Zeit. wiss. Mik.*, xvi, 1899, p. 238,) stains in concentrated aqueous solution, washes out thoroughly with water, and passes through alcohol (must be free from acid) into xylol and balsam. A metachromatic stain, granules of Nissl red, nucleoli red, all the rest yellow.

767. Tigroid Substance,—Alizarin.—See § 753.

768. Tigroid Substance, other Methods.—See Cox, *Zeit. wiss. Mik.*, xiii, 1896, p. 498; xv, p. 369; xvi, 1899, p. 101; *Anat. Hefte*, xxxi, 1898, p. 75; *Intern. Monatsschr.*, xv, 1898, H. 8; Auerbach, *Monatsschr. Psychiatrie*, iv, 1898, p. 31; *Zeit. wiss. Mik.*, xv, 1899, p. 493; Buehler, *Verh. Phys-Med. Ges. Würzburg*, xxxi, 1898, p. 285; *Zeit. wiss. Mik.*, xv, 1899, p. 351; Bielschowsky & Plien, *Neurol. Centralb.* xix, 1900, p. 1141 (concentrated cresylviolet, cold, for twenty-four hours, differentiated by alcohol).

B. *Cells and Fibres.*

769. Neurofibrils; General characters.—Nerve-cells, and the fibres into which they are prolonged, contain, in addition to the chromatic, basophilous element demonstrated by the method of Nissl, a characteristic "achromatic" element, consisting chiefly of fine, fairly refractive fibrils, which can only be seen with difficulty in the unstained state, and can only be well brought out by means of special stains. They may be fixed with *osmic acid*, and made out in thin sections of medullated nerves studied in dilute glycerin or water, and may be, to a certain extent, isolated by maceration.

The usual histological stains either leave them colourless or stain the surrounding plasma more strongly than the fibrils themselves. There may thus be produced a "negative" image of fibrils which does not really show the true neurofibrils, and being taken for them may prove a source of error. The following methods are such as have been recommended as giving true stains of the fibrils, but should not be taken to be either infallible or absolutely specific.

The gold chloride post-impregnation method of Apáthy, § 380, gives beautiful results *with certain invertebrates.*

The methylen-blue *intra vitam* method is important, see the processes of Apáthy, Dogiel, and Bethe, §§ 350–352.

770. For the purpose of making a first acquaintance with **Neurofibrils** the method of Kupffer (*Sitzb. math. Kl. Akad. wiss München*, xiii, 1884, p. 470; *Zeit. wiss. Mik.*, 1885, p. 106) may be employed. A medullated nerve is stretched on a cork and treated for twenty-four hours with 0·5 per cent. osmic acid. It is then washed in water for two hours and stained for twenty-four to twenty-eight hours in saturated aqueous solution of Säurefuchsin; after which it is washed out for from six to twelve hours (not more in any case) in absolute alcohol, cleared in clove oil imbedded in paraffin, and cut. It is said that if sections are mounted by the water method the stain will be extracted; but why not make the water *acid*, § 303?

771. Ramón y Cajal's **Silver Method for Neurofibrils** in *small and medium nerve-cells* (*Zeit. wiss. Mik.*, xx, 1904, p. 402).—Small pieces of fresh tissue are put *direct* into solution of nitrate of silver of the following strengths:

For embryos and new-born subjects, 0·75 to 0·5 per cent.

For terminal ramifications (cerebellum, etc.) and for general purposes, 1·5 per cent.

For man and large mammals, 3 per cent.; impregnates pericellular ramifications.

For invertebrates, 5 to 6 per cent; impregnates the terminal buds of Auerbach.

In general the weaker solutions give the sharper stain of neurofibrils and of nucleoli and the intra-nuclear bodies of Mann and Lenhossek, whilst the stronger ones are better for the terminal buds of Auerbach.

The preparations are to be kept for about three days (2½ for very small objects, such as spinal cord of new-born rabbit; 4 for medium-sized, such as cord or cerebellum of adult rabbit; 5 for specimens of the size of its cerebrum) in the silver. They must be kept in a stove at a temperature of 30° to 35° C. all the time. In summer, with a temperature constantly over 22° C., the stove may be dispensed with, provided that the impregnation be prolonged for two or three days more. The tissues are known to be ripe for reduction when a freshly cut surface shows a brownish-yellow coloration.

They are then washed for one or two minutes in distilled water, and put into

Pyrogallol or hydroquinon	1 to 2 grm.
Water	100 „
Formol	5 „

(The formol may be omitted).

They remain in this for twenty-four hours. They are then washed, hardened in alcohol, imbedded in celloidin (or, if not too hard, in paraffin), and sections mounted in damar.

Sections from the outer layers are too dark for study, those from the innermost too pale (if the specimens are large ones), whilst those from intermediate layers are fit for study.

The method is said to be simple, certain, and applicable to both large and small mammals and other vertebrates, to either adult or embryonic tissues, with which last it gives specially good results, and to invertebrates. It stains, besides neurofibrils, all terminal ramifications, leaves neuroglia unstained, and does not stain artefacts.

Dogiel (*Anat. Anz.*, xxv, 1904, p. 558) finds this method gives results not attainable by other means in the study of the corpuscles of Grandry. Strong solutions (2–4 or 6 per cent.) give the best results, with stoving for 4 to 6 days. Similarly Kolmer (*ibid.*, xxvi, 1905, p. 560) with epiderm of *Lumbricus*, etc.

Tellyesnicky (*Verh. Anat. Ges.*, 1904, p. 183) advises toning the sections for five to thirty minutes in 150 c.c. of water with 4 c.c. of 1 per cent. gold chloride.

772. Ramón y Cajal's **Silver Method for Neurofibrils** of *large cells*, and for *axis-cylinders* of medullated nerves (*ibid.*, p. 405).—Fresh tissue is first to be *fixed* for twenty-four hours in *alcohol of* 97 *per cent.*, then silvered as last § in 1 per cent. silver nitrate, reduced in

Hydroquinon	2 grm.
Water	100 „
Formol	5 „

(to which 0·5 gr. of anhydrous sodium sulphite may be added to quicken the reduction, which, however, will only be necessary for large specimens)

and further treated as before.

If the impregnation of inner layers should be too weak, the sections may be treated with

Sulphocyanide of ammonium	3 gr.
Hyposulphite of sodium	3 „
1 per cent. gold chloride	a few drops.

Neurofibrils of cells brown, axis-cylinders black, annular constrictions, dichotomies, and basket-fibres round cells of Purkinje demonstrated.

773. Ramón y Cajal's **Silver Method for Neurofibrils** of *motor cells* and *large connecting cells* of the bulbus, cerebellum, cerebrum, and ganglia, and for *axis-cylinders* of medullated nerves of new-born mammals (*ibid*, p. 407). As last §, but adding to the alcohol 0·5 to 1 per cent. (or for large specimens 1·5 per cent.) of ammonia, and taking the silver of 1·5 per cent. The rest as last §.

Or, instead of alcohol for hardening, you may take—

Formol	20 c.c.
Water	100″
Ammonia	0·5″

but then the objects must be washed for twelve hours in running water before silvering.

773a. The following is the latest form of a **Silver Method** of Bielschowsky (*Neurol. Centralb.*, xxii, 1903, p. 997, and xxiii, 1904, p. 387; *Zeit. wiss. Mik.*, xx, 1904, p. 462, and xxi, 1905, p. 512).—Fix, for any desired time, days or months, in 12 per cent. formol. Put for 24–48 hours (this is for retina: voluminous objects will doubtless require more) into 2 per cent. nitrate of silver. Rinse and put until brown-black into the following freshly prepared solution: to 20 c.c. of 2 per cent. nitrate of silver solution add 2 to 3 drops of 40 per cent. caustic soda solution (precipitate). Then add, with continual stirring, ammonia until the precipitate is dissolved. The solution should be clear, with a distinct smell of ammonia. Rinse again and put for 12–24 hours, or until black throughout, into 20 per cent. formol. Dehydrate, make paraffin sections, and fix on slide with Mayer's albumin. In order to have permanent preparations the sections must now be toned with gold, preferably in a bath of 10 c.c. water with 2–3 drops of 1 per cent. gold chloride and 2–3 of acetic acid. After toning put for half a minute into 5 per cent. solution of hydrosulphite, to which, if the gold bath has been an acid one, there may be added a little acid sodium sulphate ("sauerer Sulfitlauge"), one drop of the concentrated solution to 10 c.c. of water. Wash, dehydrate, clear with carbol-xylol, mount in balsam. Sections of frozen formol material may be impregnated in the same way. The method is applicable to very old formol material. It impregnates intracellular fibrils, axis-cylinders, and the "networks" of Golgi.

774. **Neurofibrils,** APÁTHY'S **Hæmatein Method** (*Mitth. Zool. Stat. Neapel,* xii, 1897, p. 712). Material may be fixed with sublimate, liquid of Zenker, picro-sulphuric acid, or any mixture that is not inimical to staining with alum hæmatoxylin, and should be preserved in 90 per cent. alcohol. Portions are stained for at least forty-eight hours in the hæmatin solution I A, § 277, and are then washed for up to twenty-four hours in *absolutely pure* distilled water, preferably suspended therein. Before the stain has become washed out of the neurofibrils, it is fixed therein by putting the preparations for three to five hours into spring water, after which they are put back for not more than two hours into distilled water, dehydrated as rapidly as possible by hanging them up in absolute alcohol, and imbedded in paraffin, or celloidin, or glycerin jelly; they must be protected from the light whilst in the chloroform through which they are passed into the paraffin, or whilst in the celloidin. Sections are made and mounted in a resin or in neutral glycerin.

This method has given splendid results with Hirudinea and some other *invertebrates,* but I believe has not yet been successful with vertebrates.

775. **Iron Hæmatoxylin.**—The methods §§ 259 or 260 sometimes stain neurofibrils very sharply, but are very uncertain. PATON (*Journ. exp. Med.,* v, 1900, p. 21) fixes pieces of cerebral cortex in sublimate (saturated with 5 per cent. acetic acid), and makes paraffin sections which are treated for an hour or two with *Tinct. ferri Rademacheri,* then stained for twenty-four hours in Apáthy's hæmatein (§ 277) and differentiated in a mixture of 1 part of anilin oil with 9 of 70 per cent. alcohol.

776. **Neurofibrils, Berlin Blue** (S. MEYER, *Anat. Anz.,* xx, 1902, p. 535).—Material is best fixed in 10 per cent. formol, then mordanted for eight to twenty days in ferrocyanide of potassium of 2½ per cent. (or this salt may be added to the fixative), then put for two to four days into iron alum (§ 260) of 10 per cent. Wash for several hours, imbed in paraffin, and mount sections in balsam. Besides neurofibrils, the sheath of Schwann and the constrictions of Ranvier are stained. Like the Golgi chrome-silver process, this method gives a *partial* impregnation, only certain elements taking on the stain, and is, therefore, likely to prove a useful succedaneum to the Golgi method.

777. Neurofibrils, Toluidin Blue (BETHE, *Zeit. wiss. Mik.*, xvii, 1900, p. 13).—Pieces of *central* nervous system (*of Vertebrates*) are fixed for twenty-four hours in nitric acid of from 3 per cent. to 7·5 per cent. strength, and brought direct into alcohol of 96 per cent. for a day or more. They are put for twelve to twenty-four hours into a mixture of one part of ammonia (of sp. gr. 0·95) with three of water and eight of 96 per cent. alcohol, then for six to twelve hours into pure alcohol; they are then put for twenty-four hours into a mixture of one part concentrated hydrochloric acid with three of water and eight to twelve of alcohol, then for ten to twelve into pure alcohol, and thence for not more than two to six hours into water. They are now mordanted for twenty-four hours in a 4 per cent. solution of ammonium molybdate, brought for twenty-four hours into alcohol, and imbedded in paraffin (*not* celloidin). Sections are seriated on albumen, then passed through xylol and alcohol into water, and "differentiated"—by which the author means washed out—with water. About 1 to 1·5 c.c. of distilled water should be poured on to the slide so as to form over the sections a layer 1·5 to 2 mm. deep, and the slide is put for two to ten minutes into a stove heated to not more than 55° to 60° C. The sections are then rinsed several times with water, a solution of one part of toluidin blue in 3000 of water is poured on to them, they are again stoved for ten minutes, rinsed with water, treated with 96 per cent. alcohol till no more colour comes away, and passed through absolute alcohol and xylol into xylol balsam.

The method is also applicable to invertebrates, for which other fixations than nitric acid are admissible, and the impregnation with the molybdate may be done on the sections instead of the uncut tissues. The results are not so certain as for vertebrates. For further details see the original, or *Encycl. mik. Technik.*, p. 934.

778. Neurofibrils.—MÖNCKEBERG and BETHE (*Arch. mik. Anat.*, liv, 1899, p. 141) recommend (for *peripheral nerves only*) the following:—Nerves are fixed in 0·25 per cent. osmic acid for twenty-four hours and bleached with bisulphite of sodium, as directed § 38, and cut in paraffin. The sections are stained on the slide for ten minutes in 0·1 per cent. solution of toluidin blue, warmed to 50° or 60° C., washed with water for one or two minutes, then treated for a few seconds or minutes with 1 per cent. solution of molybdate of ammonium. Water, alcohol, xylol, balsam.

Or the sections are first mordanted for five to ten minutes in 4 per cent. solution of molybdate of ammonium warmed to 20° or 30° C., and washed with water; then toluidin blue solution (of 0·05 to 0·1 per cent.) is poured on to the slide, which is put for five minutes into a stove at 50° to 60° C. Water, alcohol, xylol, balsam.

778a. Neurofibrils.—JORIS (*Bull. Acad. Med. Belg.*, April 30th, 1904; *Zeit. wiss. Mik.*, xxi, 1905, p. 486) gives the following as being simple and certain. Material fixed by the usual methods is put for 8 to 12 hours into a 5 per cent. solution of ammonium molybdate, then imbedded in paraffin. The sections (fixed on slides by the water method, § 200) must be washed for many hours or days in water, to remove the excess of molybdate. They are then treated for about ten minutes with a 1·5 per cent. solution of colloidal gold in water, rinsed and mounted. The stain is permanent.

The colloidal gold used was obtained from the Chemische Fabrik HEYDEN, in Radebeul-Dresden. It will dissolve in about a day.

778b. For the methods of Cox for the fibrils of spinal ganglion-cells (*Anat. Hefte*, 10, 1898; *Festschr. Niederl. Psych. Ver.*, 1896) see *Zeit. wiss. Mik.*, xiii, 1897, p. 498, or *Encycl. mik. Technik*, p. 931.

779. GOLGI'S **Intracellular Network** ("*apparato reticolare interno*"). His method for this is given § 821.

KOPSCH (*Sitzb. Acad. Wiss. Wien*, xl, 1902, p. 929; *Zeit. wiss. Mik.*, xx, 1904, p. 347) finds the net may be well demonstrated in spinal ganglion cells as follows: the ganglia are put for eight days (or exceptionally, a few more), into osmic acid of 2 per cent. and paraffin sections made. The network becomes quite black and is sharply demonstrated, unless the impregnation has been too prolonged, in which case the rest of the cell body becomes blackened also.

C. *Medullated Fibres.*

780. General Structure.—In order to demonstrate the axis-cylinder and the sheath of Schwann, the myelin may be removed. This may be done by boiling in caustic soda, and then neutralising; by boiling in a mixture of absolute alcohol and ether, and adding caustic soda; by boiling in glacial acetic acid; by boiling in fuming nitric acid, and adding caustic potash ; or by treating with *eau de Javelle* ; or (van Gehuchten, *in litt.*) the myelin may be extracted in the cold by leaving the nerves for some time in a mixture of alcohol and ether.

Or, you may tease a nerve on the slide and simply add a drop of collodion and a cover.

To dissociate nerves into their fibres, Schwalbe (*Ueb. d. Kaliberverhältnisse d. Nervenfasern*, Leipzig, 1882, p. 12 ; S. Mayer, *Anat. Anz.*, xxiii, 1903, p. 231) puts for 24 hours into osmic acid of 1 per cent., then for 24 hours at least, at a temperature of 40° C., into glycerin with hydrochloric acid (1 per cent. for frog, 3 per cent. for mammals, and for the latter 2 to 3 days in the stove). Agitate or tease on slide, the nerves separate easily into their fibres, which are found admirably preserved. (A good method for demonstrating the spiral fibre of the sympathetic cells of the frog.)

For the annular constrictions see Gedoelst, next §. See also for general instructions the *Traité* of Ranvier.

781. Neuroceratin Structures (Galli, *Zeit. wiss. Mik.*, iii, 1886, p. 467).—Small portions of ischiatic nerve are put for 18 to 20 days into solution of Müller, then for 1 or 2 days into solution of Müller diluted with 2 parts of water, then for a quarter of an hour into glycerin containing 1 or 2 drops of glacial acetic acid for each cubic centimetre, and finally (without previous washing with water) are stained for fifteen to twenty minutes in aqueous solution of China blue, washed out in alcohol cleared in essence of turpentine, and mounted in damar. Care must be taken *not to stretch* the nerve when excising it.

Platner's **Method** (*Zeit. wiss. Mik.*, vi, 1889, p. 186).—Small nerves are fixed and hardened for several days in a mixture of 1 part of Liq. Ferri Perchlor. (Ph. G., ed. 2) and 3 to 4 parts of water or alcohol, washed out in water or alcohol till no traces of iron remain in them, stained for several days or weeks in a concentrated solution of "Echtgrün" in 75 per cent. alcohol, dehydrated, imbedded, and sectioned. See also Beer, *Jahrb. Psychiatrie*, ii, 1893, 1 Heft.

Cox (*Anat. Hefte*, i, 1898, p. 75) fixes nerves in osmic acid of 2 per cent. (rabbit) or 1 per cent. (frog), washes, dehydrates, clears with bergamot oil, and mounts in balsam.

The bergamot oil dissolves out the myelin, and leaves the neuroceratin visible. It may be necessary to leave the nerves for forty-eight hours in the oil.

CORNING (*Anat. Anz.*, xvii, 1900, p. 309) studies the network in the ischiatic of the frog on sections of sublimate material strongly stained with iron hæmatoxylin.

SALA (*Verh. Anat. Ges.*, 1900, p. 176) employs the method of VERATTI for the intracellular network, § 821.

See also KAPLAN (*Arch. Psychiatr.*, xxxv, 1902, p. 825; *Zeit. wiss. Mik.*, xix, 1903, p. 508)—sections stained with Säurefuchsin and differentiated by the method of PAL.

GEDOELST (*La Cellule*, iii, 1887, p. 117) employs digestion-methods, and also (*ibid.*, v, 1889, p. 136) with advantage the following: (*a*) A nerve is treated with *liquid of* PERÉNYI, either pure or with addition of a trace of osmic acid, and examined in glycerin. By this treatment the myelin loses its excessive refractivity and the network comes out clearly. If after treatment with the liquid of Perényi the nerve be digested for some hours in 70 per cent. alcohol, still stronger images will be obtained. (*b*) Silver nitrate. Good images, but uncertain. (*c*) Treatment with a mixture of osmic acid of 1 per cent. and absolute alcohol. The network comes out black.

For the study of the *annular constrictions* Gedoelst (*op. cit.*, p. 142) fixes a nerve in extension and treats with osmic acid of 1 : 600, 800, 1000, or 2000. Solutions of 1 : 100 or stronger are *not suitable*. Examine in glycerin, or (after staining with Bismarck brown) in balsam. He also uses silver nitrate of 0·5 to 2 per cent.

For this object RAMÓN Y CAJAL employs the stain § 772.

782. Other Methods for Medullated Nerve.—RANVIER, *Traité*, p. 718, *et seq.*; REZZONICO, *Arch. per le Sci. Med.*, 1879, p. 237; TIZZONI, *ibid.*, 1878, p. 4 (a process of boiling in chloroform for an hour or two, then staining and mounting in glycerin); BOVERI, *Zeit. wiss. Mik.*, iv, 1887, p. 91; JAKIMOVITCH, *Journ. de l'Anat.*, xxiii, 1888, p. 142, or *Zeit. wiss. Mik.*, v, 1888, p. 526 (instructions for impregnating the axis-cylinder with silver, followed by reduction in formic acid and amyl alcohol); SCHIEFFERDECKER, in BEHRENS, KOSSEL, u. SCHIEFFERDECKER, *Das Mikroskop*, Bd. ii, p. 227; HUBER, *Zeit. wiss. Mik.*, x, 1893, p. 394 (stains with BENDA's safranin and Lichtgrun); RABL, *ibid.*, xi, 1894, p. 42 (the lines of Frommann are artefacts due to the silver nitrate); FISCHEL, *ibid.*, p. 48 (similar conclusion); TIRELLI, *ibid.*, xi, 1894, p. 391; SEGALL, *Journ. de l'Anat.*, xxix, 1893, p. 586; MARCHESINI, *Anat. Anz.*, xii, 1896 p. 211 (sublimate and sulphide of potassium).

CHAPTER XXXIII.

MYELIN STAINS (WEIGERT AND OTHERS).

783. Introduction.—The most important of the methods for the study of tracts of medullated nerve-fibres are the hæmatoxylin methods of Weigert.

There have been in all three methods of Weigert—the 1884 method, the 1885 method, and the 1891 method. They depend on the formation, in the tissues, of hæmatoxylin lakes which stain the myelin of nerves in a quite specific way.

The 1884 method (*Fortschr. d. Med.*, 1884, pp. 113, 190; *Zeit wiss. Mik.*, 1884, pp. 290, 564), which depends on the formation of a chrome lake, may be considered to be superseded. Not so the two others, which depend on the formation of a copper lake in addition to the chrome lake.

For a critical history of these methods see Weigert, in *Ergebnisse der Anatomie*, vi, 1896 (1897), p. 5, and in the art. "Nervenfasern, Markscheiden der," in *Encycl. mik. Technik.*

784. Weigert's **1885 Method** (*Fortschr. d. Med.*, 1885, p. 136; *Zeit. wiss Mik.*, 1885, pp. 399, 484; *Ergebnisse der Anatomie*, vi, 1896 [1897], p. 10).—The tissues are to be hardened in bichromate of potash. Weigert takes (*Ergebnisse*, p. 10) a 5 per cent. solution, and if time is an object hardens in a stove. (Other bichromate mixtures will do, *e. g.* Müller's, Kultschizky's, Zenker's; Erlicki's is not to be recommended.) The tissues are "ripe" for staining when the hardening has been carried to a certain point. They are first (*Ergebnisse*, p. 13) yellow, without differentiation of the grey matter from the white; these are unripe. Later they show the grey matter light brown, the white matter dark brown (owing to reduction of a part of the bichromate to a chrome oxide in the medullary sheaths); these are "ripe." If the hardening be continued "all the more highly oxidised chrome will pass into the lower stage of oxidation, and the tissues will become green." The tissues are then over-ripe, and cannot be used for myelin-staining without mordanting with copper or the like.

More lately (*ibid.*, p. 14) he added to the bichromate solution 2 per cent. of chrome alum or of fluoride of chromium, which hastens the hardening, so that small specimens become brown and ripe in four to five days, without stoving.

After due hardening, the preparation is imbedded by infiltration with celloidin (if desired: imbedding is not obligatory) and the celloidin block fastened on cork and hardened in the usual way. The hardened block is put for one or two days into saturated solution of neutral acetate of copper diluted with one volume of water, the whole being kept at the temperature of an incubating stove. By this treatment the tissues become green and the celloidin bluish green. The mordantage of the tissue is now terminated, and the preparation may be kept till wanted for sectioning in 80 per cent. alcohol.

Sections are made with a knife wetted with alcohol, and are brought into a stain composed of—

Hæmatoxylin	0·75 to 1 part.
Alcohol	10 parts.
Water	90 „
Saturated solution of lithium carbonate	1 part.

They remain there for a length of time that varies according to the nature of the tissues: spinal cord, two hours; medullary layers of brain, two hours; cortical layers, twenty-four hours.

They are then rinsed with water, and brought into a decolorising solution composed of—

Borax	2·0 parts.
Ferricyanide of potassium . .	2·5 „
Water	200·0 „

They remain there until complete differentiation of the nerves (half an hour to several hours), and are then *well washed* with water, dehydrated with alcohol, and mounted in balsam. They may be previously stained, if desired, with alum-carmine for the demonstration of nuclei.

The results are splendid—blue-black nerves on a golden ground. The method is applicable to the study of peripheral nerves as well as to nerve-centres, and also to the study of lymphatic glands, skin (see SCHIEFFERDECKER, *Anat. Anz.*, ii, 1887, p. 680), bile-capillaries, and other objects.

The process is applicable to tissues that have been hardened in alcohol or in any other way, provided that they be put into a solution of a chromic salt until they become brown, before mordanting them in the copper solution.

It is not necessary that the mordanting be done in bulk. MAX FLESCH (*Zeit. wiss. Mik.*, iii, 1886, p. 50) prefers (following LICHTHEIM) to make the sections first, and mordant them separately.

VASSALE (quoted from BAYON'S *Hist. Untersuchungsmeth. d. Nervensystems*, Würzburg, 1905, p. 124) first stains the sections in 1 per cent. hæmatoxylin, for three to five minutes, puts for three to five minutes into saturated solution of acetate of copper, and differentiates.

For a method for regenerating the staining solution after use, see FANNY BERLINERBLAU, *Zeit. wiss. Mik.*, 1886, p. 50, or *early editions.*

PANETH (*ibid.*, 1887, p. 213) makes the stain with extract of logwood instead of pure hæmatoxylin.

BREGLIA (*ibid.*, vii, 1890, p. 236) stains with liquid extract of logwood or Pernambuco wood.

For both of these see *early editions.*

GEROTA (*Intern. Monatsschr. Anat.*, xiii, 1896, pp. 138, 139 ; *Zeit. wiss. Mik.*, xiii, 1896, p. 315) states that the reaction can be obtained by using the copper *after* the stain, and that an alum-hæmatoxylin may be used.

785. WEIGERT'S **1891 Method** (*Deutsche med. Wochenschr.*, 42, 1891, p. 1184 ; *Zeit. wiss. Mik.*, viii, 1891, p. 392).—The material is to be hardened in bichromate and imbedded in celloidin (see last §). Then in the original form of this process Weigert proceeds as follows :

The hardened blocks of celloidin are brought into a mixture of equal parts of a cold saturated solution of neutral acetate of copper and 10 per cent. aqueous solution of potassio-tartrate of sodium ($C_4H_4O_6KNa + 4H_2O$, salt of Seignette). They are left in the mixture for twenty-four hours in an incubator. (Large specimens [pons] will require forty-eight hours, the mixture being changed for fresh at the end of twenty-four hours.) They are then brought for twenty-four hours into aqueous solution of neutral acetate of copper, either saturated or diluted with 1 volume of water, being kept as before in the incubator. They are then rinsed with water and brought into 80 per cent. alcohol, in which they may either remain till wanted or be cut after half an hour.

The object of the salt of Seignette was to prevent the pure cupric acetate from forming precipitates on the tissues. WEIGERT now (*Encycl. mik. Technik.*, 1903, p. 942) has abandoned it, finding the end is better attained by taking for the mordant a solution containing 5 per cent. of acetate of copper, 5 per cent. of acetic acid, and 2½ per cent. of fluoride of chromium, in which (as I gather) he mordants as before.

Sections are then made and stained for from four to twenty-four hours at the temperature of the room in a freshly prepared mixture of 9 vols. of (A) a mixture of 7 c.c. of saturated aqueous solution of carbonate of lithium with 93 c.c. of water, and 1 vol. of (B) a solution of 1 grm. of hæmatoxylin in 10 c.c. of alcohol (A and B may be kept in stock, but A must not be too old). The sections should be loose ones, not such as have been seriated in celloidin, and should not be thicker than 0·025 mm. The stain is poured off and the sections are washed in several changes of water poured on to them. They are then treated with 90 per cent. alcohol, followed by carbolic-acid-and-xylol mixture (for a short time only), or by a mixture of 2 parts of anilin oil with 1 of xylol, then pure xylol and xylol balsam (not chloroform balsam, which injures the stain).

It was, however, found that preparations thus made, *without differentiation*, did not keep well, and WEIGERT (*Ergebnisse d. Anat.*, iii, 1894, p. 21) reverted to the practice of differentiating with the borax-ferricyanide mixture, last §.

Lastly, he now (*Encycl. mik. Technik.*, 1903, p. 942) uses with good results a stain composed of equal parts of (A) a mixture of 4 c.c. of the officinal *Liquor ferri sesquichlorati* with 96 of water, and (B) a mixture of 10 c.c. of 10 per cent. solution of hæmatoxylin in alcohol with 90 of 96 per cent. alcohol. The two must be mixed immediately before use, and poured on to the sections, which should remain in it over night or longer, then be rinsed and differentiated as usual. This has the advantage of demonstrating very fine fibres, and of giving a colourless ground.

For difficult objects the differentiating liquid may be diluted with water, and gives better results than dilute acetic or hydrochloric acid or the like, which were formerly recommended.

Modifications of Weigert's Method.

786. Pal's **Method** (*Wein. med. Jahrb.*, 1886; *Zeit. wiss. Mik.*, iv, 1887, p. 92; *Med. Jahrb.*, 1887, p. 589; *Zeit. wiss. Mik.*, 1888, p. 88).—This is a *chrome-lake* process. You proceed at first as in Weigert's process, but *omitting the copper bath,* and you stain as in Weigert's process. After staining in the hæmatoxylin solution the sections are washed in water (if they are not stained of a deep blue a trace of lithium carbonate must be added to the water). They are then brought for twenty to thirty seconds into 0·25 per cent. solution of permanganate of potash, rinsed in water, and brought into a decolouring solution composed of—

Acid. Oxalic. pur.	1·0
Potassium Sulphite * (Kalium Sulfurosum [SO_3K_2])	1·0
Aq. Dest.	200·0

In a few seconds the grey substance of the sections is decolourised, the white matter remaining blue. The sections should now be well washed out, and may be double-stained with Magdala red or eosin, or (better) with picro-carmine or acetic-acid-carmine.

For further details see the papers quoted, or Behrens, Kossel, and Schiefferdecker's *Das Mikroskop,* i, p. 199.

Pal's process gives brilliant results, the ground of the preparations being *totally colourless.* But it has the defect that the differentiation is more rapid than is desirable. The whole process of differentiation only lasts some seconds, so that an error of judgment of only a few seconds may entirely vitiate the result.

Weigert (*Ergebnisse,* vi, p. 21) considers that for very thick sections the process is superior to his own. But it is not so safe for very fine fibres, and is not applicable to his collodion series method; each section must be treated separately.

Marcus stains by the Pal method sections of material hardened in *formalin,* as described § 745.

Gudden (*Neurol. Centralb.*, xvi, 1897, p. 24) makes celloidin sections of material hardened in 5—10 per cent. formol followed by alcohol, treats them for ten hours with 0·55 per cent. chromic acid, rinses with water, and treats with 80 per cent. alcohol, then stains by the method of Pal, adding to the hæmatoxylin a few drops of dilute nitric acid (MINNICH).

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* Not "sulphide," as erroneously given in Mercier's *Les Coupes du Système Nerveux Central,* p. 190.

TSCHERNYSCHEW and KARUSIN (*Zeit. wiss. Mik.*, xiii, 1896, p. 354), stain for twenty-four hours in the *hæmatoxylin* of KULTSCHITZKY, § 788.

So also PAVLOW, *ibid.*, xxi, 1904, p. 14, taking the permanganate twice as strong as Pal.

See also DÖLLKEN, *ibid.*, xv, 1899, p. 444, or *previous editions.*

787. KAISER (*Neurol. Centralb.*, xii, 1893, pp. 364, 368; *Zeit. wiss. Mik.*, xi, 1894, p. 249) hardens first in liquid of Müller, then for eight days in *liquid of* MARCHI (§ 797), mordants sections for five minutes with sesquichloride of iron (1 part to 1 of water and 3 of 70 per cent. alcohol), stains, and differentiates with Pal's liquid. For details see *early editions.*

BOLTON (*Journ. of Anat. and Phys.*, xxxii, 1898, p. 245) makes sections of formalin material, and mordants them for a few minutes in 1 per cent. osmic acid, or for a few hours in iron-alum or ammonium molybdate, stains in KULTSCHITZKY's hæmatoxylin (next §), and differentiates by Pal's process.

Similiarly WYNN, *ibid.*, 1900, p. 381.

LASLETT (*Lancet*, 1898, p. 321; *Journ. Roy. Mic. Soc.*, 1898, p. 600) mordants in liquid of Marchi (1 week), makes sections, stains by KULTSCHITZKY's method, and differentiates by PAL's.

788. KULTSCHITZKY (*Anat. Anz.*, 1889, p. 223, and 1890, p. 519) has given two modifications of WEIGERT's method, of which the following is the later: Specimens are hardened for one or two months in *solution of Erlicki,* imbedded in celloidin or photoxylin, and cut. Sections are stained for from one to three hours, or as much as twenty-four, in a stain made by adding 1 grm. of hæmatoxylin dissolved in a little alcohol to 100 c.c. of 2 per cent. *acetic acid.* They are washed out in saturated solution of carbonate of lithia or soda.

Differentiation is not necessary, but by adding to the carbonate of lithia solution 10 per cent. of a 1 per cent. solution of red prussiate of potash, and decolorising therein for two or three hours or more, a sharper stain is obtained. After this the sections are well washed in water and mounted in balsam.

WOLTERS (*Zeit. wiss. Mik.*, vii, 1891, p. 466) proceeds as Kultschitzky, except that he stains in a solution kept warm by placing it on the top of a stove kept at 45° C. for twenty-four hours, after which time the sections are dipped in solution of Müller, and differentiated by the method of Pal.

Similarly Kaes (*ibid.*, viii, 1891, p. 388; *Neurol. Centralb.*, 1891, No. 15).

789. Mitrophanow (*Zeit. wiss. Mik.*, xiii, 1896, p. 361) mordants photoxylin sections for at least twenty-four hours at 40° C. in a mixture of equal parts of saturated aqueous solution of acetate of copper and 90 per cent. alcohol, stains for ten minutes in Kultschitzky's hæmatoxylin, and differentiates with Weigert's ferricyanide.

790. Berkley's **Rapid Method** (*Neurol. Centralb.*, xi, 9, 1892, p. 270; *Zeit. wiss. Mik.*, x, 1893, p. 370).—Slices of tissue of not more than two and a half millimetres in thickness are hardened for twenty-four to thirty hours in *mixture of Flemming*, at a temperature of 25° C., then in absolute alchol, then imbedded in celloidin and cut. After washing in water the sections are put overnight into a saturated solution of acetate of copper (or they may be simply warmed therein to 35° to 40° C. for half an hour). They are then washed, and stained for fifteen to twenty minutes in the fluid given below, warmed to 40° C., allowed to cool, and differentiated for one to three minutes in Weigert's ferricyanide liquid, which may be diluted if desired with one third of water. Water, alcohol, bergamot oil, xylol-balsam.

The stain is made as follows: 2 c.c. of saturated solution of carbonate of lithia are added to 50 c.c. of boiling water and the solution boiled for two minutes more, when 1½ to 2 c.c. of 10 per cent. solution of hæmatoxylin in absolute alcohol are added.

Liquid of Flemming had been used before by Friedmann (*Neuro. Centralb.*, 1885).

790a. Benda's **Rapid Method** (*Berlin klin. Wochenschr.*, No. 32, 1903).—Sections of *formol material* by the freezing process are stained for twenty-four hours in Boehmer's hæmatoxylin, differentiated with Weigert's ferricyanide, and mounted in balsam. Only recommended for peripheral nerves, or for preliminary examination of the central nervous system.

791. Streeter (*Arch. Mik. Anat.*, lxii, 1903, p. 734) stains *small nerve-centres* in bulk (after mordanting in

Weigert's bichromate and fluoride mixture, § 784) with Weigert's hæmatoxylin (four to six days), washes for a couple of days in 70 per cent. alcohol, makes paraffin sections, and differentiates them by the method of Weigert or Pal.

792. Gallein.—Aronson (*Centralb. med. Wiss.*, 1890, p. 577) stains sections of material hardened in liquid of Erlicki or Müller (these must be mordanted with acetate of copper) for twelve to twenty-four hours in a solution of 3 to 4 c.c. of Gallein (Grübler & Co.) in 100 c.c. of water with 20 of alcohol and three drops of concentrated solution of carbonate of soda. They are then differentiated by the method of Weigert, or Pal, or with a mixture of a few drops of saturated solution of chlorinated lime to a watch-glass of water, then brought into concentrated solution of carbonate of soda or lithia until they become red, and are mounted in balsam (clear with oil of origanum). Nerve-fibres red. A second stain with methylen blue may follow (best after differentiating with permanganate). Similarly Schrötter (*Centralb. allg. Path.*, xiii, 1902, p. 512).

793. Schrötter (*Neurol. Centralb.*, xxi, 1902, p. 338; *Zeit. wiss. Mik.*, xix, 1903, p. 381) also stains sections for two to three hours in a 5 per cent. solution of sulphalizarinate of soda, to which is added a few drops of 5 per cent. oxalic acid (enough to give an orange tint), then differentiates until no more colour comes away, in carbonate of soda solution of $\frac{3}{1000}$ strength, and mounts in balsam. Myelin red, on a colourless ground.

794. Toluidin Blue.—Harris (*Philadelphia Med. Journ.*, May 14th, 1898) stains sections (of material hardened as for Weigert's stain) for several hours in a 1 per cent. solution of toluidin blue in 1 per cent. borax solution, and differentiates in saturated aqueous solution of tannic acid. Similarly, but with methylen blue, in a complicated way, Fraenkel, *Neurol. Centralb.*, xxii, 1903, p. 766 (*Zeit. wiss. Mik.*, xx, 1904, p. 345).

795. Other Modifications or Similar Methods.—Flechsig, *Arch. Anat. Phys., Phys. Abth.*, 1889, p. 537; *Zeit. wiss. Mik.*, vii, 1890, p. 71; Breglia, *ibid.*, p. 36; Rossi, *ibid.*, vi, 1889, p. 182; Mercier, *ibid.*, vii, 1891, p. 480; Haug, *ibid.*, p. 153; Walsem, *ibid.*, xi, 1894, p. 236; Robertson, *ibid.*, xiv, 1897, p. 80 (*Brit. Med. Journ.*, 1897, p. 651); Hill, *Brain*, lxxiii, 1896; *Phil. Trans.*, 184B, 1894, p. 399.

Strong (*Journ. Comp. Neur.*, xiii, 1903, p. 291) finds bichromate of copper the best mordant; also describes an iron-hæmatoxylin process.

Other Myelin Stains.

796. Osmic Acid (Exner, *Sitzb. Akad. Wiss. Wien*, 1881, lxxxiii, p. 151; Bevan Lewis, *The Human Brain*, p. 105). A portion of brain, not exceeding a cubic centimetre in size, is placed in ten times its volume of

1 per cent. osmic acid, replaced by fresh after two days. In from five to ten days it is cut (best without imbedding). The sections are treated by caustic ammonia (20 drops to 50 c.c. of water), which clears up the general mass of the brain substance, leaving medullated fibres black, and are examined in glycerin. The method shows very fine fibres, but the preparations are not permanent.

797. MARCHI'S **Method (for Degenerate Nerves)** (*Rivista sperim. di Freniatria*, 1887, p. 208; *Zeit. wiss. Mik.*, ix, 1893, p. 350).—Small pieces of tissue are hardened for a week in solution of Müller, and then put for a few days into a mixture of 2 parts solution of Müller and 1 part 1 per cent. osmic acid solution. Sections are cut, best without imbedding, and mounted in balsam. The treatment with the chrome salt deprives the medullary sheath of normal fibres of the faculty of impregnating with osmium, whilst the (fatty) degeneration products in deceased sheaths retain that faculty. In consequence the sheaths in normal nerves acquire a yellow coloration, those of degenerated tracts a black one.

For the study of degenerate nerve-tracts the method of MARCHI has an advantage over that of WEIGERT, in that it gives *positive* images of the degenerated elements, Weigert's process only giving negative ones.

For a critical review of this method and its modifications see WEIGERT, in *Ergebnisse der Anatomie*, vii, 1897 (1898), pp. 1—8.

The method has been applied to tissues that have been hardened in formol; but this, according to WEIGERT, does not seem recommendable.

FINOTTI (*Virchow's Arch.*, cxliii, 1896, p. 133) makes sections of material that has been in liquid of Müller for not more than a few weeks or months, and puts them for four to ten hours into a freshly prepared mixture of one or two parts of 1 per cent. osmic acid and one part of a concentrated solution of picric acid in one third alcohol (the mixture must be protected from light during the reaction). For peripheral nerves, myelin (normal), black.

ORR (*Journ. Path. and Bact.*, vi, 1900, p. 387; *Journ. Roy. Mic. Soc.*, 1900, p. 399) treats with a mixture of 8 c.c. of 2 per cent. osmic acid, and 2 c.c. 1 per cent. acetic acid, which greatly increases the penetration.

BUSCH (*Neurol. Centralb.*, xvii, 1898, p. 476; *Zeit. wiss. Mik.*, xv, 1899, p. 373) puts formol-hardened material for five to seven days into a solution of one part osmic acid, three of iodate of sodium, and 300 of water. Same stain as Marchi's, but more penetrating and sharper.

798. AZOULAY'S **Osmic Acid Method** (*Anat. Anz.*, x, 1894, p. 25).—(A) Sections of material that has been for several months in liquid of Müller are put for five to fifteen minutes into solution of osmic acid of 1 : 500 or 1 : 1000 strength. Rinse with water and put them for two to five minutes into a 5 or 10 per cent. solution of tannin, warming them therein over a flame till vapours are given off, or in a stove at 50° to 55° C. Wash for five minutes in water, double-stain if desired with carmine or eosin, and mount in balsam. Thin sections are necessary to ensure good results. If they should be too thick it will be necessary after staining to differentiate by PAL'S process, or by *eau de Javelle* diluted with 50 vols. of water. (B) Material that has been in an osmic mixture (liquid of Flemming, of Marchi, or of Golgi). Sections as before, then the tannin bath, warming for three to ten minutes, and the rest as before.

799. HELLER AND GUMPERTZ (*Zeit. wiss. Mik.*, xii, 1896, p. 385) give for peripheral nerves, and HELLER (*op. cit.*, xv, 1899, p. 495) for central nervous system, the following: The material is hardened in liquid of Müller. Sections (by the celloidin method if desired) are put into 1 per cent. osmic acid (twenty-four hours at 37° C.) for peripheral nerves, ten minutes (or thirty at the normal temperature) for central. They are treated with pyrogallic acid (a photographic developer will do) till the nerves are black, then with a violet-coloured solution of permanganate of potash till the sections become brown, then with 2 per cent. oxalic acid till they become yellow-green. Wash out well between each operation; the preparations are then permanent. Mount in glycerin or balsam.

Similarly, TELJATNIK (*Neurol. Centralb.*, 1897, p. 521); ROBERTSON (*Brit. Med. Journ.*, 1897, p. 651; *Journ. Roy. Mic. Soc.*, 1897, p. 175), the material being previously mordanted with Weigert's chrome-alum-copper fluid for neuroglia; and ORR, *loc. cit.*, § 797. See also ROSSOLIMO & BUSCH, *Zeit. wiss. Mik.*, xiv, 1897, p. 55.

800. RAMÓN Y CAJAL (*Trab. lab. Biol. Madrid*, ii, 1903, p. 93; *Zeit.

wiss. Mik., xx, 1904, p. 458) describes a method of treating Marchi material that seems to me to be of inadmissible complexity.

801. Iron.—ALLERHAND (*Neurol. Centralb.*, xvi, 1897, p. 727; *Encycl. mik. Technik.*, p. 944) puts sections of Müller material for fifteen minutes into warm 50 per cent. solution of *Liquor ferri sesquichlorati*, then for an hour or two into 20 per cent. tannin solution (old and brown). They are then differentiated by the method of PAL, taking, however, the liquids twice as strong.

An iron-alum process is described by STRONG in *Journ. comp. Neurol.*, xiii, 1903, p. 291.

802. Silver Nitrate.—VASTARINI-CRESI (*Att. Accad. Med.-Chir. Napoli*, 1, 1896) hardens in formol, cuts thick sections, washes them with 40 per cent. alcohol, puts them in the dark into 1 per cent. solution of nitrate of silver in alcohol of 40 per cent. to 70 per cent., then washes thoroughly.

Similarly, MOSSE (*Arch. mik. Anat.*, lix, 1901, p. 401) impregnating bichromic material with 1 per cent. solution of argentamin, and reducing in 10 per cent. pyrogallic acid, and differentiating by the method of PAL.

803. Gold Chloride for Peripheral Nerves (FREY, *Arch. Anat. Phys. Anat. Abth. Supp.*, 1897, p. 108. See *Grundzüge*, LEE & MAYER, p. 421).

804. Polarisation.—Myelin can sometimes be detected in *fresh material* by the polariscope (see AMBRONN & HELD, *Ber. Math. Phys. Ges. Wiss. Leipzig.*, 1895, p. 37). They examined teased preparations of peripheral nerves fresh in normal salt solution, or thick sections of nerve-centres cut with the freezing microtome.

See also GAD & HEYMANS, *Arch., Anat. Phys., Phys. Abth.*, 1890, p. 531.

Myelin-and-axis-cylinder Stains.

805. Palladium (PALADINO, *Rendic R. Accad. Scienze*, Napoli, iv, 1890, p. 14, and 1891 [1892], p. 227; *Zeit. wiss. Mik.*, vii, 1890, p. 237, and ix, 1892, p. 238).—Pieces of material hardened in bichromate, chromic acid, or corrosive sublimate, and not more than 5 to 8 mm. in thickness, are put for two days into a large quantity (at least 150 to 200 c.c. for each piece) of 0·1 per cent. solution of chloride of palladium (see § 82). They are next put for twenty-four hours into a solution of iodide of potassium of 4 : 100 strength, of which a relatively small volume should be taken; otherwise the iodide of palladium, which is rapidly formed in the tissues, may be again extracted by the liquid (small pieces of tissue should not remain in it for more than one or two hours). Dehydrate; imbed, if necessary, in paraffin by the chloroform method; mount in balsam.

Later (*Boll. Accad. Med. Roma*, xix, 1893, p. 256; *Arch. Ital. Biol.*, xx, 1894, p. 40) he first dehydrates the pieces, then puts them in an incubator for an hour into absolute alcohol and benzol, an hour in pure benzol, and finally twenty-four hours in absolute alcohol, which removes the myelin. They are then put for a week into chloride of palladium of 1 to 2 per cent., one to two days into 4 per cent. iodide of potassium, and are lastly passed through alcohol into celloidin.

806. Methylen Blue.—SAHLI (*Zeit. wiss. Mik.*, 1885, p. 1) stains sections of tissue hardened in bichromate to the degree required for Weigert's hæmatoxylin process for several hours in concentrated aqueous solution of methylen blue, rinses with water, and stains for five minutes in saturated aqueous solution of Säurefuchsin. If now the sections be rinsed with alcohol and brought into a liberal quantity of water, the stain becomes differentiated, axis-cylinders being shown coloured red and the myelin sheaths blue.

The same author (*loc. cit.*, p. 50) also gives the following: Sections of material hardened as before are stained for a few minutes or hours in the following liquid:

Water	40 parts.
Saturated aqueous solution of methylen blue	24 ,,
5 per cent. solution of borax	16 ,,

(Mix, let stand a day, and filter.)

The sections are then washed either in water or alcohol until the grey matter can be clearly distinguished from the white, are cleared with cedar oil, and mounted in balsam. Nerve-tubes blue, ganglion-cells greenish, nuclei of neuroglia blue.

807. Säurefuchsin.—FINOTTI (*op. cit.*, § 797) stains strongly in Delafield's hæmatoxylin, then for a few seconds in concentrated solution of picric acid, then in 0·5 per cent. Säurefuchsin, and treats with alkaline alcohol (caustic potash).

OHLMACHER (*Journ. Exper. Med.*, ii, 1897, p. 675) stains sections on the slide for one minute with anilin-water gentian, then for a few seconds in a solution of 0·5 per cent. of Säurefuchsin in saturated solution of picric acid diluted with one volume of water, washes well with water, differentiates with alcohol and clove oil, and mounts in balsam.

808. Safranin.—Method of ADAMKIEWICS (*Sitzb. k. Akad. Wiss. Wien.*

Math. Naturw. Kl., 1884, p. 245 ; *Zeit. wiss. Mik.*, 1884, p. 587).—Sections (of spinal cord hardened in liquid of Müller for not less than one month and not more than three) are washed first with water, then in water acidified with a little nitric acid, and stained in concentrated solution of *safranin*. They are then treated with alcohol and clove oil till no more colour comes away, and are brought back again into water, washed in water acidified with acetic acid, stained in *methylen blue*, and cleared as before. Myelin red, nuclei violet.

NIKIFOROW (*Zeit. wiss. Mik.*, v, 1888, p. 338) impregnates with gold chloride or other metallic salt after the safranin stain.

Similarly CIAGLINSKI (*Zeit. wiss. Mik.*, viii, 1891, p. 19) and STROEBE (*ibid.*, x, 1893, p. 336), the former employing safranin followed by anilin blue, whilst the latter first stains with anilin blue, then differentiates with alcohol containing a very little caustic potash, and after-stains with safranin.

For NISSL's **Congo red** method see *Zeit. wiss. Mik.*, iii, 1886, p. 398.

809. Kodis (*Arch. mik. Anat.*, lix, 1901, p. 211) fixes for one or two days in saturated solution of cyanide of mercury, hardens in 10 per cent. formol, and stains sections of frozen material by Heidenhain's iron hæmatoxylin method, § 260.

CHAPTER XXXIV.

AXIS-CYLINDER AND DENDRITE STAINS (GOLGI AND OTHERS).

810. Introduction.—There are three chief methods for the *anatomical* (§ 733) study of axis-cylinders and protoplasmic nerve-cell processes, viz. the methylen-blue *intra-vitam* method, the bichromate-and-silver method of GOLGI, and the bichromate-and-sublimate method of GOLGI. The methylen-blue method having been given in Chapter XVI, there remain to be described here the two methods of GOLGI and some other methods suitable for the same or similar purposes.

811. The Methods of GOLGI. There are two methods of GOLGI, viz. the **Bichromate and Nitrate of Silver Method** and the **Corrosive Sublimate Method.**

The bichromate and nitrate of silver method has been worked out by GOLGI in *three* forms—the *slow* process, the *rapid* process, and the *mixed* process.*

The rapid process is the one that is the most in use at the present time for researches into the distribution and relations of axis-cylinders and dendrites; it may be taken to be the classical method of inquiry into the finer relations of the neurons in *hardened* tissue.

General characters of the impregnation.—The preparations have not in the least the appearance of stains, and are even very different in aspect from the impregnations obtained on fresh tissue by the ordinary methods of impregnating with nitrate of silver or chloride of gold. The impregnation is a *partial* one, by which is meant that of all the elements, whether nervous or not, that are present in a preparation, only a limited number are coloured. That is the peculiar quality—not by any means the defect, but rather the advantage—of the method. For if all the elements present were coloured equally, with the great intensity with which they take the colour in this method, you would not be able to see the wood for the trees; in fact, you would hardly be able to distinguish any detail at all in the preparations. But Golgi's method selects from among the elements present a small number which it stains with a great intensity and very completely—that is to say, throughout a great length, so that they are both very clearly separated from those elements that have remained uncoloured, and also can be followed out for a great distance.

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* In a recent text-book, the *Leitfaden* of RAWITZ, the sublimate method is called "the slow method of GOLGI," and the bichromate and silver nitrate method is given under the form of the slow process, and called "the rapid method of GOLGI." That is a very "nice derangement of epitaphs" indeed. RAWITZ further attributes the rapid method to RAMÓN Y CAJAL, which is equally erroneous. Similar confusions are made by MERCIER in his *Coupes du Système Nerveux Central*, and by POLLACK and other authors. Valuable accounts of the silver method have been given by v. LENHOSSÉK in his *Feinere Bau d. Nervensystems*, 2nd edit., 1895, and by KALLIUS in the art. "Golgische Methode" in the *Encycl. mik. Technik.*, 1903.

Axis-cylinders are *generally* impregnated only so long as they are *not medullated*. In the adult the method stains nerve-cells and their processes, so far as these are not myelinated; but if it be wished to impregnate the axis-cylinders of the cerebro-spinal axis the method is best applied to embryos or new-born animals at a time when the fibres have not become surrounded by their sheath of myelin.

There is no other method which will allow cell-processes to be followed out for such great distances. But the method does not demonstrate at the same time the histological detail of other tissues that may be present in the preparations, and all cytological detail is lost. It is *par excellence* a *special* method.

Nervous tissue is not the only thing that is impregnated in these preparations; neuroglia, connective tissue, fibrils, etc., are impregnated, and the method has been applied with success to the study of such things as bile-capillaries, gland-ducts, and the like. Both on account of this character, and on account of the capriciousness with which the impregnation takes hold of only certain elements of the preparations, care must be exercised in the interpretation of the images obtained. A further source of possible error is found in the fact that the method frequently gives precipitation-forms of the silver salt that simulate dendrites and other structures (see Friedlaender in *Zeit. wiss. Mik.*, xii, 1895, p. 168, and the plate in the following number).

The method has been applied with success to the tissues of Invertebrates—Insects, *Lumbricus, Tubifex, Helix, Limax, Distomum, Astacus, Actinida, etc.*

The details of the method have been considerably modified at the hands of various workers, the most important modification being that of the "double" or "intensified" impregnation of Ramón y Cajal.

The method has been described at length by Golgi in the *Archives Italiennes de Biologie,* t. iv, 1883, p. 32 *et. seq.*, and vii, 1886, p. 15 *et. seq.* The following account is from the latter paper. The earlier form of the method should not be followed.

812. Golgi's **Bichromate and Nitrate of Silver Method,** *SLOW Process* (*loc. cit.*, p. 17).—(*a*) *The hardening.*—This must be done in a bichromate solution. Either pure bichromate of potash may be employed, or liquid of Müller (the reaction can be obtained with liquid of Erlicki, but it is not to be recommended). The normal practice is to take bichromate of potash, beginning with a strength of 2 per cent., and changing this frequently for fresh solutions of gradually increased strength, 2½, 3, 4, and 5 per cent. The tissue should be *as fresh as possible;* though satisfactory results may be obtained from material taken twenty-four to forty-eight hours after death.* *It should be in pieces of not more than 1 c.cm. or 1½ c.cm. in size.*

The most difficult point of the method consists in *hitting off the exact degree of hardening* in the bichromate that should be allowed before passing to the next stage of the process, the silver-bath. In summer good results may be obtained after fifteen to twenty days, and the material may continue in a favourable state for impregnation up to thirty, forty, or fifty days. In cold weather good results can seldom be obtained under a month: when obtained, the material may continue to give good results up to two, three, and even four months of hardening. The only way to make sure is to pass trial portions of the tissue at intervals into the silver-bath, in summer frequently, in winter every eight or ten days, and observe whether the reaction is obtained.

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* Material that has been hardened in formol may also be used. See § 819 (Gerota and Bolton), and v. Lenhossék's *Feinere Bau d. Nervensystems*, p. 23.

Good results are obtained by *injecting* the organs with the hardening fluid (2·5 per cent. bichromate). See § 734.

Stoving at a temperature of 20° to 25° C. is useful for abridging the hardening, but there is risk of over-hardening; and Golgi thinks the results are never quite so delicate as after hardening in the cold.

(*b*) *Impregnation.*—As soon as the pieces of tissue have attained the proper degree of hardening, they are brought into a bath of nitrate of silver. The usual strength of this bath is 0·75 per cent., but 0·50 per cent. may be taken for material that has not been quite enough hardened, and solutions of 1 per cent. may be used for material that has been slightly over-hardened. The solution *may* be acidified (see Ramón y Cajal, § 823).

A relatively large quantity of solution should be taken for the bath.

The moment the pieces of tissue are put into the silver-bath an abundant yellow precipitate of chromate of siver is formed. This of course weakens the bath *pro tanto*. It is therefore well, before putting the pieces into the final silver-bath, to first wash them well in a weaker silver solution, until on being put into a fresh quantity of it no further precipitate is formed. Used solutions will do for this purpose. The final silver-bath in general needs no further attention, unless it be that sometimes, in the case of tissues that have taken up a great deal of bichromate of potash, the solution may after six to ten hours become somewhat yellow, in which case it should be changed for fresh.

It is not necessary to keep the preparations in the dark during the impregnation bath; in winter it is well to keep them in a warm place.

The time necessary for impregnation by the silver is from twenty-four to forty-eight hours. The normal time is from twenty-four to thirty hours, forty-eight being quite exceptional. By this is meant that the reaction is not obtained in less time, but tissues may remain in the bath without hurt for days, weeks, or months.

(*c*) *Preservation.*—As soon as a trial has shown that a sufficiently satisfactory impregnation has been obtained the pieces are brought into alcohol. The alcohol is changed two or three times, or even more, until it remains transparent even after the preparations have been two or three days in it; for in view of good preservation it is necessary that the excess of nitrate of silver should be washed out from them thoroughly.

Sections are now made (see §§ 825–827). They are to be washed very thoroughly in three or four changes of absolute alcohol. They are then cleared, first in creosote, in which they should remain only a few minutes, then in oil of turpentine, in which they should remain for ten to fifteen minutes (they may remain there for days without hurt). They are then mounted in damar (rather than in balsam), *and without a cover.* Preparations mounted under covers in the usual way always go bad sooner or later, whilst those that are mounted without a cover keep very well, *especially if they be kept in the dark.* Golgi states that he has a large number that have kept without change for nine years.

As a general rule *thick* sections (50 to 60 μ or more) show much more than thin ones, but do not seem to keep so well.

The order in which the elements of tissues impregnate is generally—first, axis-cylinders, then ganglion cells, and lastly neuroglia cells.

813. Golgi's **Bichromate and Nitrate of Silver Method,** *RAPID process* (*op. cit.*, p. 33). Small pieces of very fresh tissue are thrown into the following mixture:

Bichromate solution of 2 to 2·5 per cent. strength	8 parts.
Osmic acid of 1 per cent. strength	2 ,,

The hardening being much more rapid than with the slow process, the tissues will begin to be in a fit state for taking the silver impregnation from the second or third day; in the next following days they will be in a still more favourable state, but the favourable moment does not last long; the faculty of impregnation soon declines, and is generally quite lost by the tenth or twelfth day.

The silver impregnation is conducted exactly as in the slow process, and sections are prepared and mounted in the same manner.

There is this difference, that the impregnated material cannot be preserved for any length of time in alcohol, but *must not remain for more than two days in it.* But it may be kept in the *silver solution* until wanted for sectioning. According to VAN GEHUCHTEN (*La Cellule,* vi, 1890, p. 405), material may be kept for six months in the silver, *with advantage,* showing abundant reductions where none were found after forty-eight hours. But it *must be kept in the dark.*

The following notes as to the proper duration of the hardening process in different cases are taken from the papers quoted and other sources, most of which may be found in v. LENHOSSÉK, *op. cit.*, p. 23.

Spinal cord of chick, from the sixth to the tenth day of incubation—twelve to forty-eight hours in the mixture (up to the fifth day the embryos may be treated whole, later the vertebral column should be dissected out and cut into two or three segments; it need not be opened). The spinal column of new-born rats and mice should be treated in the same way, and remain in the mixture for twenty-four hours (for spinal ganglia), or for two to six days for the cord itself. (The encephalon of these subjects may be treated in just the same way, without being dissected out.)

VON LENHOSSÉK (*op. cit.*, p. 10) recommends for human (fœtal) cord two to three days for neuroglia, three to five for nerve-cells, and five to seven for nerve-fibres and collaterals.

Cerebellum of new-born subjects, three to five days in the mixture.

Cerebral cortex of young subjects, two to three days (Mice), or as much as five (Rabbit, Cat); cortex of adults, eight to fifteen days. The most favourable region of the brain is the *cornu Ammonis*, especially in the Rabbit.

Retina—twenty-four to forty-eight hours in the mixture, then "double" impregnation (§ 816A).

Sympathetic—double or triple impregnation.

Epidermis of *Lumbricus*—three to six days in the hardening mixture, and two in the silver, or double impregnation if necessary. SMIRNOW makes the mixture of equal parts of 5 per cent. bichromate and 1 per cent. osmic acid, and leaves it in for five to twenty-eight days, and one to two days in the silver (0·75 per cent.).

As a general rule, the younger the subject, the shorter should the hardening be. If it has been too short, sections will have a brownish-red opaque aspect, with precipitates, and irregular impregnation of cells and fibres. If it has been too long, the ground will be yellow, without precipitates, but with no impregnated elements, or hardly any.

This process has the advantage of great rapidity, and of sureness and delicacy of result, and is the one that has found the most favour with other workers. But for methodical study of any given part of the nervous system Golgi himself prefers the following:

814. Golgi's **Bichromate and Nitrate of Silver Method,** *MIXED Process* (*op. cit.*, p. 34).—Fresh pieces of tissue are put for periods varying from two to twenty-five or thirty days into the usual bichromate solution (§ 812). Every two or three or four days some of them are passed on into the osmio-bichromate mixture of the rapid process, hardened therein for from three or four to eight or ten days, and finally impregnated with silver, and subsequently treated exactly as in the rapid process.

The reasons for which Golgi prefers this process are—the certainty of obtaining samples of the reaction in many stages of intensity, if a sufficient number of pieces of tissue have been operated on; the advantage of having at one's disposition a notable time—some twenty-five days—during which the tissues are in a fit state for taking the silver, and the possibility of greatly hastening the process whenever desired by simply bringing the pieces over at once into the osmic mixture; lastly, a still greater delicacy of result, especially remarkable in the demonstration of the "functional" or nervous process of nerve-cells.

815. Critique of Golgi's **Method.**—The above-described methods have been found extremely valuable in the most various departments of nervous anatomy. They have given brilliant results in the study of peripheral nerves and their origins or terminations, and in the study of the relations of fibres and cells in the central nervous system. It has been found at the same time that they have the defect of considerable uncertainty in the production of the desired reaction and in the preservation of the stain.

Golgi's method is apparently (but this is by no means certain) based on the formation in the tissues of a precipitate of some salt of silver which is brown by reflected light, but appears black by transmitted light. The problem is to preserve this precipitate in the tissues free from chemical or molecular change. And the problem is not an easy one; without special precautions the preparations will not resist the processes necessary for imbedding, will not always resist those necessary for merely mounting in balsam, and even then may easily "go bad" after they have been mounted for a short time.

A critical review of the Golgi method by WEIGERT may be found in *Ergebnisse der Anatomie*, v, 1895 (1896), p. 7. He thinks the precipitate certainly consists of *a* silver chromate, but that we cannot say *which*.

The method has also been critically studied by HILL (*Brain*, part 73, 1896, p. 1). He thinks the stain depends on the formation of a "reduced salt (subsalt) of silver," not of a silver chromate, and that the reduction takes place, not in the nervous fibrils, but in the liquid or semi-liquid "neuroplasm" with which they are bathed. He finds the impregnated material will stand imbedding in celloidin for many days.

AZOULAY (*Comptes Rend. Soc. Biol.* [10], i, 1894, p. 839) has followed the process under the microscope, and holds that it is due to a crystallisation of chromate of silver in the tissues.

KALLIUS (*Encycl. mik. Technik.*, p. 466) thinks that an albumino-chromate of silver is formed.

Modifications concerning the Impregnation of the Tissues.

816. RAMÓN Y CAJAL (*Zeit. wiss. Mik.*, vii, 1890, p. 332) gives 3 per cent. as the strength of the bichromate in the mixture for the rapid process, and in numerous other places has given it as 3·5 per cent. This latter strength has been adopted by most of the workers who use the rapid process, and the mixture containing this proportion of bichromate is generally known as RAMÓN Y CAJAL's mixture.

816a. RAMÓN Y CAJAL'S **Double-Impregnation Process** (*Trab. Lab. Hist. Med. Barcelona*, 1891; *Zeit. wiss. Mik.*, ix, 1892, p. 239).—After hardening for three days (embryos of fowl) in the osmium-bichromate mixture the preparations are put for thirty-six hours into nitrate of silver solution (0·5 to 0·75 per cent.). They are then brought back for a day or two into the same osmium-bichromate mixture, or into a weaker one containing only two parts of osmic acid solution to 20 of the bichromate. After treatment with this they are washed quickly with distilled water, and put for a second time into the silver solution for thirty-six to forty-eight hours. It is important to hit off the proper duration of the first impregnation in the bichromate. If it has been too long (four days) or too short (one day), the second impregnation will not succeed. In this case a third impregnation must be resorted to, the objects being again treated with the weak osmium-bichromate mixture, and afterwards again with the silver solution.

This modification of the original process is, perhaps, the most important that has hitherto been made.

817. KALLIUS (*Anat. Hefte*, iii, 1894, p. 531) states that he has often found it advantageous to employ bichromate of *ammonia* or of *soda* instead of the bichromate of potash, and to perform all the reactions in the dark.

818. BOEHM, and afterwards OPPEL (*Anat. Anz.*, v, 1890, p. 143, and vi, 1891, p. 165) take, instead of bichromate of potash (slow process), the one a 0·5 per cent. solution of *chromic acid* (forty-eight hours), the other a solution of *neutral chromate* of potash of from 0·5 per cent. to as much as 10 per cent. This is for liver.

BERKLEY (*Anat. Anz.*, 1893, p. 772) fixes pieces of liver for fifteen to thirty minutes in warm half-saturated solution of picric acid, and hardens for forty-eight hours in a stove in the dark in a "sunned" (§ 357) mixture of sixteen parts 2 per cent. osmic acid and 100 parts saturated solution of bichromate.

819. Formaldehyde Mixtures.—STRONG (*Anat. Anz.*, x, 1895, p. 494) states that formaldehyde can with advantage be substituted for the osmic acid in the osmio-bichromic mixture of GOLGI's rapid process. He adds from 2·5 to 5 per cent. of formalin to the (3·5 to 5 per cent.) bichromate solution.

The advantage is stated to be that the stage of hardening favourable for impregnation lasts longer; in other words, the formaldehyde bichromate does not over-harden.

DURIG (*ibid.*, p. 659) obtained the best results by means of 3 per cent. bichromate solutions containing 4 to 6 per cent. of formaldehyde, hardening therein for three days, and then performing double impregnation by RAMÓN Y CAJAL's process.

FISH (*Proc. Amer. Mic. Soc.*, xvii, 1895, p. 319) takes:

Formalin	2 c.c.
3 per cent. bichromate . . .	100 ,,

leaving the tissues three days in this liquid and three days in the silver nitrate ($\frac{3}{4}$ per cent.).

Or, with advantage:

Liquid of Müller	100 c.c.
10 per cent. formalin . . .	2 ,,
1 per cent. osmic acid . . .	1 ,,

Odier (*La Rachicocainisation,* Genève, 1903, p. 27) takes two parts of undiluted formalin, instead of the 10 per cent.

The formalin and bichromate mixtures should be kept in the dark. It is well only to make them up *at the instant of using* them. Odier finds these mixtures afford a more abundant impregnation, with fewer precipitates.

Kopsch (*Anat. Anz.,* xi, 1896, p. 727) takes 4 parts of 3·5 per cent. bichromate solution, and 1 of commercial formaldehyde solution, and after twenty-four hours transfers to pure 3·5 per cent. bichromate. He finds that by this means precipitates are almost entirely avoided.

Gerota (*Intern. Monatsschr. Anat.,* xiii, 1896, p. 108) first hardens (brain) for a week or two in 5 to 10 per cent. formol solution, then puts small pieces for three to five days into 4 per cent. bichromate, then into the silver.

Similarly Bolton (*Lancet,* 1898, p. 218; *Journ. Roy. Mic. Soc.,* 1898, p. 244).

Schreiber (*Anat. Anz.,* xiv, 1898, p. 275) obtained good results (on appendages of Crustacea which were impervious to the osmic mixture) with mixtures of five parts 2·5 per cent. bichromate to one of 4 per cent. formaldehyde, or one part 2·5 per cent. bichromate to two of 5 per cent. formaldehyde, the specimens remaining for one day in the first, for two days in the second.

Similarly Duboscq (*Arch. z. Exper.,* 1899, p. 483), warming the mixture to 40° C.

van Gehuchten (*in litt.*), and other observers, *have not obtained good results* with formaldehyde.

820. Acetic Aldehyde.—Vassale and Donaggio (*Monitore Zool., Ital.,* vi, 1895, p. 82) harden pieces of at most 1 cm. in thickness for fifteen to twenty days in a mixture of five parts of aldehyde with 100 of 3 to 4 per cent. bichromate, changing the fluid after a few days, as soon as it has become dark. The rest as Golgi.

821. Veratti's **Liquid.**—For the study of Golgi's "endocellular network" Golgi (*Verh. Anat. Ges.,* xiv, 1900, p. 174) uses a mixture due to Veratti, consisting of:

5 per cent. bichromate . . .	30 parts.
0·1 per cent. platinum chloride . .	30 ,,
1 per cent. osmic acid . .	15 to 30 ,,

and after hardening therein puts (for a time varying from a few hours to ten days) into a mixture of 1 part of bichromate of 3 or 4 per cent. with two of saturated solution of sulphate of copper, and thence into silver nitrate of 0·8 to 1 per cent. See further *Verh. Anat. Ges.*, xiv Vers., 1900, p. 174.

822. Reviving Over-hardened Tissues.—Tissues that have been too long in the osmium-bichromate mixture will no longer take on the silver impregnation. They can, however, be revivified and made to impregnate in the following manner, due to GOLGI, and published by SACERDOTTI (*Intern. Monatsschr.*, xi, 1894, p. 326). They are washed in a half-saturated solution of acetate of copper until they no longer give a precipitate, and are then put back again for five or six days into the osmium-bichromate mixture. Sections of the impregnated material give remarkably fine images, and will bear mounting in thickened oil of cedar under a cover.

More recently (*Cinquantenaire Soc. Biol.*, 1899, p. 514) Golgi puts for some hours or days into a mixture of equal parts of bichromate of 2 to 3 per cent. and sulphate of copper of 4 to 5 per cent., or into the cupric mixture given last §.

823. Modifications of the Silver Impregnation.—RAMÓN Y CAJAL (*Rev. trim. Hist.*, No. 2, 1888, note) found the addition of a very little formic acid to the silver bath facilitated reduction. According to VAN GEHUCHTEN (*La Cellule*, vii, 1891, p. 83) 1 drop of the acid should be added to 100 c.c. of the silver. But I gather that the practice is now generally abandoned.

BERKELEY (*Johns Hopkins Hosp. Rep.*, vi, 1897, p. 1; *Journ. Roy. Mic. Soc.*, 1898, p. 242) impregnates, after hardening in the osmiobichromate, in a freshly prepared solution of two drops of 10 per cent. phosphomolybdic acid to 60 c.c. of 1 per cent. silver nitrate, which in winter should be kept at a temperature of about 26° C.

HILL (*op. cit.*, § 815) takes instead of silver nitrate a $\frac{3}{4}$ per cent. solution of silver *nitrite*, with 0·1 per cent. of formic acid added.

GUDDEN (*Neurol. Centralb.*, xx, 1901, p. 152) takes the lactate of silver (sold as "actol") and finds it much more penetrating.

FAJERSTAJN (*ibid.*, p. 98) uses ammonio-nitrate (see p. 243) in a complicated way.

824. Avoidance of Precipitates.—GOLGI's process frequently gives rise to the formation at the surface of the preparations of voluminous precipitates that are destructive of the clearness of the images. Sehrwald (*Zeit. wiss. Mik.*, vi, 1889, p. 456) finds that this can be avoided by putting the tissues into gelatin solution before bringing them into the silver-bath. A 10 per cent. solution of gelatin in water may be made. The tissues are imbedded in this, in a paper imbedding box, with the aid of a little heat (the gelatin melting at a sufficiently low temperature), and are brought therein into the silver-bath. After the silvering the gelatin is removed by warm water saturated with chromate of silver.

Martinotti wraps the tissue simply in blotting-paper, but this does not appear to be efficacious.

Athias takes wafer-papers.

Ramón y Cajal covers tissues with a layer of congealed blood, which need not be removed before cutting, or with collodion, or peritoneal membrane. See § 851.

Modifications concerning the Preservation of the Preparations.

825. Cutting and Mounting.—Many most elaborate methods have been proposed with the object of fixing the stain so that the preparations may bear imbedding in paraffin and the sections bear mounting under a cover. None of them have met with much favour.

An elaborate discussion (for which see *previous editions*) between Sehrwald (*Zeit. wiss. Mik.*, vi, 1890, p. 443), Samassa (*ibid.*, vii, 1890, p. 26), and Fick (*ibid.*, viii, 1891, p. 168) furnishes the net practical result that *watery fluids* should be avoided as much as possible during the after-treatment, and that sections should either be mounted without a cover, or on a cover raised free of contact with the slide by means of wax feet or the like, or, for study, inverted over the aperture of a hollowed-out wooden slide; or that the balsam of the mount should be rendered perfectly anhydrous by careful heating on the slide, with the section in it, until it immediately sets hard on cooling, before the cover is applied.

This last method is also recommended by Huber (*Anat. Anz.*, vii, 1892, p. 587).

Odier (*Rech. expér. sur les Mouvements de la Cell. nerv.*, Genève, 1898, p. 17) finds preparations will bear a cover if not treated with any alcohol of less than 96 per cent. after silvering.

SALA (*Zeit. wiss. Zool.*, lii, 1891, p. 18; *Zeit. wiss. Mik.*, viii, p. 389), in a paper written in Golgi's laboratory, finds Greppin's method (§ 826) not merely useless, but hurtful. And he thinks that SEHRWALD'S process for imbedding the material in paraffin with the object of getting very thin sections is a mistake. The chief quality of GOLGI'S process is that it admits of the following of nerve-cell processes for a *very great distance*. Evidently this cannot be done with very *thin* sections. It is better simply to wash the preparations taken from the silver-bath with water, fix them to a cork with gum, put the whole into alcohol for a few hours to harden the gum, and cut with a microtome without imbedding.

The majority of workers seem to abide by these results, and to consider that the fixation methods shortly given in the next four sections are, to say the least, superfluous.

826. GREPPIN'S **Process** (*Arch. Anat. Entw. Anat. Abth.*, 1889, Supp., p. 55).—After silvering (slow process) sections are made with a freezing microtome and treated for thirty to forty seconds with 10 per cent. solution of hydrobromic acid, and may then be well washed in several changes of water and mounted under a cover in the usual way. Further details in *previous editions*.

827. OBREGIA'S **Process** (*Virchow's Archiv*, cxxii, 1890, p. 387).—Sections of silvered material are made, either without imbedding, or after imbedding either in paraffin or celloidin, care being taken in either case not to use alcohol of a lower grade than 94 or 95 per cent. They are brought from absolute alcohol into a mixture of eight to ten drops of 1 per cent. solution of gold chloride with 10 c.c. of absolute alcohol, which should be prepared half an hour beforehand and exposed to diffused light until the sections are placed in it, when it should be put into the dark. After fifteen to thirty minutes therein, according to their thickness, the sections are quickly washed in 50 per cent. alcohol, then in water, then treated for five or ten minutes with 10 per cent. solution of hyposulphite of soda. They are lastly washed well with water, and may then be mounted at once in balsam under a cover, or if desired may be previously stained with carmine or hæmatoxylin, or Pal's modification of Weigert's process, or the like.

828. KALLIUS (*Anat. Hefte*, ii, 1892, p. 271) has worked out the following process. Take 20 c.c. commercial hydroquinone developing solution and 230 c.c. distilled water (the hydroquinone solution may be made up with 5 grms. hydroquinone, 40 grms. sodium sulphite, 75 grms. carbonate of potassium, and 250 grms. distilled water). At the instant of using, further dilute the solution with one third to one half its volume of absolute alcohol, and put the sections into it for several minutes; they become dark grey to black. As soon as reduction is complete the sections are put for ten to fifteen minutes into 70 per cent. alcohol, then brought for five minutes into solution of hyposulphite of soda (about 10 parts to 50 of water), and thence into a large quantity of distilled water, where they should remain for twenty-four hours or more. Lastly, dehydrate in the usual way and mount under a cover. After-staining with carmine, etc., may be employed.

829. ZIMMERMANN'S **Process** (*Arch. mik. Anat.*, lii, 1898, p. 554).—Sections are brought, after silvering, from alcohol into a mixture of 1 part physiological salt solution and 2 parts 96 per cent. alcohol. They must be kept in motion therein for ten to fifteen minutes, after which they are brought into alcohol of 75 to 96 per cent. in a bright light, until they have become dark. It is said that sections so treated will bear mounting under a cover.

The Sublimate Method.

830. GOLGI'S **Bichromate and Sublimate Method** (*Archivio per le Scienze Mediche*, 1878, p. 3; *Archives Italiennes de Biologie*, iv, 1883, p. 32; vii, 1886, p. 35).—This method consists of two processes: 1, hardening in bichromate; 2, treatment with bichloride of mercury.

For hardening, use either a solution progressively raised in concentration from 1 per cent. to 2½ per cent., or Müller's solution. It is *best* to take small pieces of tissue (not more than 1 to 2 c.c.), large quantities of liquid, and change the latter frequently, so as to have it always clear. But the reaction can be obtained with much larger pieces, *even entire hemispheres*. In this case the brain should at first be treated by repeated injections of the liquid, so as to ensure as rapid a permeation of the interior as possible. Fifteen to twenty days' immersion will suffice, or even six to eight, but twenty to thirty should be preferred, and an immersion of several months is not injurious.

The tissues when hardened are passed direct from the bichromate into 0·5 per cent. solution of bichloride of mercury. An immersion of eight to ten days therein is necessary in order to obtain a complete reaction through the whole thickness of the tissues (or for entire hemispheres two months or more). During the bath the bichromate will diffuse out from the tissues into the bichloride, which must at first be changed every day, and later on as often as it becomes yellow. At the end of the reaction the preparations will be found decolourised, and offering the aspect of fresh tissue. They may be left in the bichloride for any time.

In *Rendiconti R. Ist. Lombardo di Sci. Milano*, 2, xxiv, 1891, pp. 594, 656 (see *Zeit. wiss. Mik.*, viii, 3, 1891, p. 388), Golgi says that for the study of the " diffuse nervous reticulum " of the central nervous system the best results are obtained by keeping the preparations in 1 per cent. sublimate for a very long time, two years being not too much in some cases.

The reaction may be said to have begun by the time the tisssues are nearly decolourised. From that time onwards sections may be made day by day and examined, and those which it is desired to preserve may be mounted.

Before mounting, the sections that have been cut must be repeatedly washed with water (if it be wished to mount them permanently), otherwise they will be spoilt by the formation of a black precipitate. (In the last place quoted Golgi says that after washing they may be toned by putting them for a few minutes into a photographic fixing-and-toning bath, after which it is well to wash them again, and stain them with some acid carmine solution.) Mount in balsam or glycerin; the latter seems the better preservative medium.

The elements acted on are—(1) The ganglion cells, with all their processes and ramifications of the processes. These are made more evident than by any other process except the bichromate and silver-nitrate process. (2) Nuclei, which is not the case with the silver process. (3) Neuroglia cells. But the reaction in this case is far less precise and complete than that obtained by the silver process. (4) The blood-vessels, and particularly their muscular fibre cells.

The method is said to give *good* results only with the cortex of the cerebral convolutions, hardly any results at all with the spinal cord, and very scanty results with the cerebellum. And, on the whole, it shows nothing more than can be demonstrated by the silver-nitrate method, but it is superior to it in that the reaction can always be obtained with perfect *certainty* in a certain time; that the preparations can be perfectly preserved by the usual methods; and that large pieces of tissue can be impregnated.

See also Flatau in *Arch. mik. Anat.*, xlv, 1895, p. 158.

The method is recommended by Blochmann (*Biol. Centralb.*, xv. 1895, p. 14) for the nervous system of Cestodes.

Modifications of Golgi's Bichromate and Sublimate Method.

831. Pal* (*Gazz. degli Ospitali*, 1886, No. 68) finds that if sections made by this process be treated with solution of sodium sulphide, a much darker stain is obtained. Sections may then advantageously be double-stained with Magdala red.

Golgi's method may be combined with Weigert's nerve stain (see Pal. *Wien. med. Jahrb.*, 1886; *Zeit. wiss. Mik.*, v, 1887, p. 93).

832. Flechsig's modifications, see *Arch. Anat. Phys.*, *Physiol. Abth.*, 1889, p. 537; *Zeit. wiss. Mik.*, vii, 1890, p. 71.

833. Cox (*Arch. mik. Anat.*, xxxvii, 1891, p. 16) finds the sublimate and bichromate may be used *together*, and give a uniform impregnation. He used a fluid consisting of 20 parts 5 per cent. bichromate, 20 parts 5 per cent. sublimate, 16 parts 5 per cent. simple chromate of potash, and 30 to 40 parts of water. (The chromate should be diluted with the water before adding it.) The mixture should be as little acid as possible. The duration of the impregnation is from two to three months. There is considerable difficulty in preserving sections, which must be made with a freezing microtome, alcohol being avoided, treated for an hour or two with 5 per cent. solution of sodium carbonate, and mounted without a cover, in a medium composed of—gum sandarac, 75 gr.; camphor, 15; oil of turpentine, 30; oil of lavender, 22·5; alcohol, 75; castor oil, 5–10 drops. For examination, add a drop of castor oil, and cover.

Dr. A. Sanders, on the other hand, writes me (June, 1898) that the stain keeps very well if the sublimate be well removed by washing in many changes of alcohol, and the tissues passed through alcohol and ether into celloidin, and the sections mounted in chloroform-balsam under a cover. I think the statement is correct as far as regards the preservations of the stain; but the preparations quickly develop opaque granules that are very unsightly.

For the very complicated platinum-substitution processes of Robertson and Macdonald see *Journ. Ment. Sci.*, xlvii, 1901, p. 327; or *Journ. Roy. Mic. Soc.*, 1902, p. 501.

Other Methods.

833a. Ramón y Cajal's **Silver Methods** have been given, §§ 772 and 773.

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* *Erratum*—"Tal," *loc. cit.*

834. ZIEHEN'S **Gold and Sublimate Method** (*Neurol. Centralb.*, x, 1891, p. 65; *Zeit. wiss. Mik.*, viii, 1891, p. 385).—Small pieces of fresh material are thrown into a large quantity of a mixture of 1 per cent. sublimate solution and 1 per cent. chloride of gold solution in equal parts. They remain therein for at least three weeks, preferably for several months (up to five), by which time they will have become of a metallic red-brown colour. They are gummed on cork and sectioned without imbedding. The sections are treated either with Lugol's solution (§ 88) diluted with four volumes of water, or with dilute tincture of iodine, until duly differentiated, then washed and mounted in balsam. The result is a bluish-grey impregnation; both medullated and non-medullated nerve-fibres are stained, also nerve and glia cells and their processes.

835. APÁTHY'S **Gold Method** has been given (§ 380).

836. GERLACH'S **Bichromate and Gold Process** has been given, § 378.

For a complicated **Gold Method** of RAMÓN Y CAJAL, see *Rev. trim. Micr.*, v, 1900, p. 95; or *Zeit. wiss. Mik.*, xix, 1902, p. 187.

837. For UPSON'S exceedingly complicated **Gold and Iron and Vanadium Methods** see MERCIER, in *Zeit. wiss. Mik.*, vii, 1891, p. 474; or in his *Coupes du Système Nerveux Central*, p. 234; or *early editions.*

838. For FAJERSZTAJN'S complicated **Silver Method** see *Neurol. Centralb.*, xx, 1901, p. 98; or *Zeit. wiss. mik.*, xviii, 1901, p. 214. For that of BIELSCHOWSKI, see *Neurol. Centralb.*, xxi, 1902, p. 579; or *Zeit. wiss. Mik.*, xix, 1903, p. 370. A simpler form of this has been given, § 773 *a*.

839. KROHNTHAL'S **Lead Sulphide Impregnation** (*Neurol. Centralb.*, xviii, 1899, No. 5; *Zeit. wiss. Mik.*, xvi, 1899, p. 235) consists in treating tissues first with formate of lead and then with hydric sulphide. The formate is prepared by dropping formic acid slowly into solution of acetate of lead. White crystals of formate of lead are abundantly formed; the mother liquor is filtered off, and the crystals are dissolved to saturation in water. The solution is mixed with an equal volume of 10 per cent. formol; pieces of brain or spinal cord are put into the mixture for five days, and are then brought direct into a mixture of equal parts of 10 per cent. formol and hydric sulphide solution. After five days therein they are passed through successive alcohols, imbedded in celloidin, cut, and the sections mounted in xylol-balsam under a cover. They seem to be quite permanent. Nerve-cells as well as nerve-fibres are impregnated. The impregnation is a very complete one.

Corning (*Anat. Anz.*, xvii, 1900, p. 108) hardens the tissues with 10 per cent. formol before bringing them into the formol-formate mixture, and so obtains better results. He obtains his formate of lead direct from Merck (*Plumbum formicicum*). He thinks the celloidin imbedding injurious, and prefers to cut without imbedding. He prefers to clear sections with clove oil. The method appears to him particularly useful for the medulla oblongata, with which the Golgi method does not succeed. Other details *loc. cit.*

840. Methylen Blue for Central Nervous System (Semi Meyer, *Arch. mik. Anat.*, xlvi, 1895, p. 282, and xlvii, 1896, p. 734).—Meyer has obtained good results (for the *central* nervous system, not for the peripheral) by means of *subcutaneous* injection. *Large* quantities of solution must be injected. A young rat will require at least 5 c.c. of 1 per cent. solution; a rabbit of a few weeks about 40 c.c. But it is better to employ stronger solutions, 5 to 6 per cent. The total dose should be given in several portions, at intervals of one to several hours. It is not necessary to wait till death by intoxication has taken place, and after a suitable interval the subject may be killed. It is not necessary to expose the organs to the air for the sake of "oxydising" the stain. They should be thrown direct into the bath of Bethe, § 352. The liquid ought to be well cooled before use. The preparations should remain in it till the next day.

Ramón y Cajal (*Rev. trim. Micr.*, Madrid, i, 1896, p. 123; *Zeit. wiss. Mik.*, xiv, 1897, p. 92) stains by "propagation" or "diffusion." The brain is exposed (rabbit) and the cortex is divided into slices of a couple of millimetres thickness by means of a razor. The slices are then covered on both sides either with finely powdered methylen blue, or with a saturated solution of the same, the slices are replaced in their natural positions, the brain-case is replaced for half an hour, after which the slices are removed and fixed for a couple of hours with Bethe's ammonium molybdate, washed, hardened for three or four hours in a mixture of 5 parts 1 per cent. platinum chloride, 40 parts formol, and 60 parts water, further treated for a few minutes with platinum chloride in alcohol (1 in 300), and if small enough imbedded in paraffin. The sections should be dehydrated with alcohol containing 0·3 per cent. of platinum chloride, and may be cleared with xylol or bergamot oil and mounted. The stain is stronger and *more complete* than that of the other methylen blue methods.

841. MAGINI'S **Zinc Chloride Process** (see *Boll. Accad. Med. di Roma*, 1886 ; *Zeit. wiss. Mik.*, 1888, p. 87, or *early editions*).

842. MONTI'S **Copper Process**, see *Atti. R. Accad. Lincei Roma, Rendic.*, v, 1889, p. 705 ; *Zeit. wiss. Mik.*, vii, 1890, p. 72.

842a. Other Methods.—Anthracen Ink (Leonhardi's, obtainable from Grübler) is used in a complicated way by KAPLAN, *Arch. Psychiatr.*, xxxv, 1902, p. 825 (*Zeit. wiss. Mik.*, xix, 1903, p. 510).—STRAHUBER, *Centralb. allg. Path.*, xiii, 1901, p. 422 (*Zeit. wiss. Mik.*, xviii, 1902, p. 482) (pathological). MALLORY'S phospho-molybdic hæmatoxylin, see § 286. SAHLI'S methods, see § 806.

And see also under **Neurofibrils**, §§ 771 *et seq.*

CHAPTER XXXV.

NEUROGLIA, AND NERVE-END ORGANS.

Neuroglia.

843. INTRODUCTION.—Neuroglia cells may be isolated by teasing, and may be stained in many ways (see RANVIER, *Traité*, p. 1063), by osmic acid, hæmatoxylin, carmine, orcein. But by far the best method for the study of the forms and relations both of ependyma cells and astrocytes is the **Bichromate-and-silver Impregnation** of GOLGI; the best material being that which has been for not more than two or three days in the osmio-bichromic mixture.

This method, however, does not tinctorially differentiate between neuroglia-cells and nerve-cells, and is of no use for the purpose of mapping out tracts of neuroglia as a whole. The following methods serve for this. They are such as either stain neuroglia quite specifically, leaving all other tissues unstained (WEIGERT) or such as stain it in a different tone to other tissues. WEIGERT's process stains the processes of the cells (his "fibres") intensely, whilst leaving the cell-body unstained, and in consequence, if exclusively followed, may lead to erroneous conclusions.

844. WEIGERT'S **Specific Neuroglia Stain** (WEIGERT'S *Beitr. zur Kenntniss der normalen menschlichen Neuroglia,* Frankfurt-a-M., 1895; and his art. "*Neurogliafärbung*" in *Encycl. Mik. Technik,* 1014).—Pieces of *very fresh* tissue of *not more than* half a centimetre in thickness are put for at least four days into 10 per cent. formol. They are then mordanted for four or five days in an incubating stove (or for at least eight days at the temperature of the laboratory) in a solution containing 5 per cent. of neutral acetate of copper, 5 per cent. of acetic acid, and 2½ per cent. of chrome alum, in water. Add the alum to the water, raise to boiling point, and add the acetic acid and the acetate, powdered (or [*Encycl.*, p. 1019] instead of the chrome alum, you may take chromium fluoride, which obviates the necessity of boiling). If prepared, the mordant may be dissolved in the formol solution, so that the hardening and mordanting are done at the same time.

After the mordanting the tissues are washed with water, dehydrated, imbedded in celloidin, and sectioned. The sections are treated for ten minutes with a one third per cent. solution of permanganate of potash, and well washed in water. They are then treated for two to four hours with a solution of "Chromogen." "Chromogen" is a naphthalin compound prepared by the Hoechst dye manufactory. The solution of "Chromogen" to be used is prepared as follows: 5 per cent. of "Chromogen" and 5 per cent. of formic acid (*of* 1·20 *sp. gr.*) are dissolved in water, and the solution carefully filtered. To 90 c.c. of the filtrate are added 10 c.c. of 10 per cent. solution of sodium sulphite.

After this bath, the sections are put till next day into a saturated (5 per cent.) solution of Chromogen. (Instead of the Chromogen treatment, you may simply treat the sections with PAL's potassium sulphite solution, § 786, and the results will be nearly as good.)

They are next carefully washed and submitted to the stain. The stain is a modification of WEIGERT's *fibrin stain*, § 721. Instead of saturated aqueous solution of methyl violet, you take a warm saturated solution in 70 per cent. or 80 per cent. alcohol, decant it after cooling, and add to it 5 per cent. of a 5 per cent. aqueous solution of oxalic acid. The iodine should be a *saturated* solution in potassic iodide of 5 per cent., and should only be allowed to act for a moment. And instead of treating with pure anilin, you take a mixture of equal parts of anilin and xylol. This is afterwards removed from the sections by means of pure xylol, and they are mounted in balsam, or, preferably, turpentine-colophonium, § 454. The staining is best done on the slide, the stain being poured on and off.

WEIGERT's method, I believe, *only* gives good results with the *human subject*.

MALLORY (*Journ. Exper. Med.*, 1897, p. 532) fixes tissues for four days in 10 per cent. solution of formalin, then for four to eight in saturated solution of picric acid (or for the same time in a mixture of the two), then mordants for four to six days at 37° C. in 5 per cent. solution of bichromate of ammonia, makes sections (celloidin) and stains them in Weigert's fibrin stain.

Storch (*Virchow's Arch.*, clvii, 1899, p. 127; *Zeit. wiss. Mik.*, xvi, 1900, p. 475), instead of mordanting the material in bulk with the copper fluid, first makes celloidin sections.

Bartel (*Zeit. wiss. Mik.*, xxi, 1904, p. 18) first makes *paraffin* sections and treats them with all the reagents without removing the paraffin, until they have passed the anilin-xylol mixture, which should consist of 1 of anilin to 10 of xylol (or more), and be allowed to act for twelve to twenty-four hours.

See also Aguerre and Krause, *Arch. mik. Anat.*, clii, 1900, p. 509; and Rubaschkin, *ibid.*, cliv, 1904, p. 575.

845. Benda's Alizarin Stain (*Neurol. Centralb.*, xix, 1900, p. 796, and his art. *"Neurogliafärbung"* in *Encycl. Mik. Technik*, p. 1025) is as follows: The material is to be fixed with alcohol, and further treated with nitric acid, etc., as directed for Centrosomes, p. 355, and paraffin sections are made and fixed to slides and the paraffin removed. They are then mordanted and stained as directed under (*b*), p. 355, and differentiated and mounted as there described.

Besides this, Benda also recommends hardening and making paraffin sections as described, then staining with the modified Weigert stain given for central corpuscles under (*a*), p. 355; or, staining with Heidenhain's iron-hæmatoxylin, § 260, and differentiating with 2 per cent. iron-alum or Weigert's borax-ferricyanide mixture.

846. Mallory's **Phospho-tungstic Hæmatoxylin** (*Journ. Exp. Med.*, v, 1900, p. 19; *Zeit. wiss. Mik.*, xviii, 1901, p. 178).—Tissues to be fixed, mordanted, and cut as directed under Mallory, § 844. The sections are put for a quarter of an hour into 0·5 per cent. solution of permanganate of potash, washed and put for a quarter of an hour into 1 per cent. solution of oxalic acid, well washed, and stained for twelve to twenty-four hours or more in the following solution:

Hæmatoxylin	0·1
Water	80·0
10 per cent. solution of (Merck's) phospho-tungstic acid	20·0
Peroxide of hydrogen (U.S. Ph.)	0·2

(Dissolve the hæmatoxylin, add the acid, then the peroxide; the stain will keep).

Wash, dehydrate in 95 per cent. alcohol, clear with origanum oil, mount in xylol-balsam. Axis-cylinders and nerve-cells pink, neuroglia blue. To get a more isolated stain of neuroglia, the sections should be brought for five to twenty minutes, after staining, into a 30 per cent. alcoholic solution of dry sesquichloride of iron. Neuroglia and fibrin blue, the rest colourless.

847. ANGLADE and MOREL (*Rev. Neurol.*, ix, 1901, p. 157) harden in a mixture of 3 parts of liquid of FOL (§ 46), with 1 of 7 per cent. sublimate solution, dehydrate with alcohol followed by aceton, make paraffin sections and stain in saturated aqueous solution of Victoria blue, heated till it steams, rinse with liquid of Gram (§ 299), differentiate with xylol 1 part, anilin 2 parts, and mount in balsam. Simple, *applicable to lower animals,* and gives very sharp images.

848. Säurerubin.—KULTSCHITZKY (*Anat. Anz.*, viii, 1893, p. 357) stains paraffin sections (of material hardened in his copper liquid, § 59), either for five to ten seconds with a mixture of 1 grm. Säurerubin (Rubin S.), 400 c.c. 2 per cent. acetic acid, and 400 c.c. saturated solution of picric acid, or for half an hour in a mixture of 3 to 5 c.c. of the above stain with 100 c.c. of 96 per cent. alcohol, and washes out well with alcohol.

849. YAMAGIWA (*Virchow's Arch.*, clx, 1900, p. 358) hardens very small pieces of tissue for a month or more in liquid of Müller, makes celloidin sections, stains for twelve hours in saturated alcoholic solution of eosin, then for four to six in saturated solution of anilin blue in water, and differentiates in dilute alcohol with a very little caustic potash. Water, alcohol, origanum oil, balsam. Neuroglia red, axis-cylinders blue.

*Retina.**

850. Fixation and Hardening.—For section-cutting, the retina *of small eyes* is best prepared by fixing the entire unopened bulb with osmium vapour. According to RANVIER (*Traité*, p. 954) you may fix the eye of a triton (without having previously opened the bulb) by exposing it for ten minutes to vapour of osmium. The sclerotic being very thin in this animal, such a duration of exposure is generally sufficient. Then divide it by an equatorial incision, and put the posterior pole for a few hours into one third alcohol.

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* Besides the sources quoted in the text, see SELIGMANN, *Die mikroskopischen Untersuchungsmethoden des Auges*, Berlin, S. Karger (Karlstrasse 13), 1899; GREEF, *Anleitung zur mikr. Untersuch. d. Auges*, Berlin, Hirschwald, 1898; and the Art. "Sehorgau" in *Encycl. mik. Technik.*, 1903.

Somewhat larger eyes, such as those of the sheep and calf, may be fixed in solutions without being opened. But it is generally the better practice to make an equatorial incision, and free the posterior hemisphere before putting it into the liquid.

The older practice was to use strong solutions of pure osmic acid; but most of the best recent work has been done with chromic mixtures.

Dr. Lindsay Johnson tells me that he now gets the best results by fixing the globe over the steam of a 1 per cent. osmic acid solution raised to the temperature at which vapour is seen to be given off (but not to boiling point), for five minutes in the case of human adults, or for one to three minutes in the case of human infants, all monkeys and small mammals, as in them the sclerotics are very thin. As soon as the sclerotic is felt to be firm to the touch, it should be opened by a small nick with a razor just behind the ciliary body; or if the eye be that of an adult, the cornea and lens may be removed. The eye is then put for twelve hours into the mixture, § 49; it is then washed in running water, and suspended in a large volume of 2·5 per cent. bichromate of potash for two days, then passed gradually through successive alcohols, beginning with 20 per cent., and ending with absolute, taking five days from first to last.

Other hardening liquids, however, also give good results, provided that the fixation by the osmic acid has been properly performed: amongst them *liquid of Flemming*, and that of Müller. Formaldehyde mixtures he does *not* recommend.

Leber (*Münch. med. Wochenschr.*, xli, 30, 1894; *Zeit. wiss. Mik.*, xii, 1895, p. 256) confirms Hermann's observation concerning eyes (see p. 75). He advises a solution of formol 1, water 10. After a few days' hardening in this, the eyes may be cut through, it is said, without derangement of the parts. The retina lies flat, and is at least as well preserved as with solution of Müller. The eyes may be passed without hurt direct into successive alcohols; the vitreous will shrink a little, but less rather than more than after solution of Müller. I doubt the correct cytological preservation of the elements by this process.

See also Hippel (*Arch. f. Ophthalm.*, xlv, 1898, p, 286; *Zeit. wiss. Mik.*, xvi, 1899, p. 79), who finds that formol fixes the lens badly, the retina well, so far at least as the absence of folds from shrinkage is concerned; and HERZOG (*Arch. mik. Anat.*, lx, 1902, p. 517).

851. Staining.—For general views I recommend iron-hæmatoxylin, followed by Säurefuchsin or Picro-Säurefuchsin, or preceded by Bordeaux; or Kernschwarz, followed by safranin, § 386, and the Ehrlich-Biondi stain, § 306.

The **methylen-blue** *intra-vitam* stain has given valuable results; see the methods of DOGIEL, §§ 350 *et. seq.*

But the most important method is the **bichromate-and-silver** impregnation of GOLGI, first applied to this object by TARTUFERI (*Intern. Monatsschr.*, 1887). This author employed the rapid process. So also RAMÓN Y CAJAL (*La Cellule*, ix, 1893, p. 121, and numerous other papers), under the form of the double-impregnation process, § 816. To avoid the formation of precipitates on the tissues, which is here particularly important, he covers the retina, before silvering, with a piece of peritoneal membrane, or a thin layer of collodion. Or, better, he *rolls* the retina (*op. cit.*, p. 130). After removing the vitreous, the retina is cut away around the papilla with a punch or fine scalpel, and separated from the choroid. It is then rolled up (after being cut into quadrants or not), so as to form a solid block. This is painted with 2 per cent. celloidin, which is allowed to dry for a few seconds, and the whole is put into the bichromate mixture, and further treated as a solid mass of tissue.

GOLGI'S **sublimate impregnation** (COX'S form) has also been successfully employed by KRAUSE and RAMÓN.

The bichromate-and-silver method serves for the study of the fibres of Müller and neuroglia cells, as well as neurones. Weigert's neuroglia stain has been employed, but does not seem to have given good results.

LENNOX (*Arch. f. Ophthalm.*, xxxii, 1; *Zeit. wiss. Mik.*, iii, 1886, p. 408) has used Weigert's hæmatoxylin method.

KUHNT (*Jen. Zeit. Naturw.*, Bd., xxiv, H. 1, 1889, p. 177) employs Pal's modification. Similarly SCHAFFER (*Sitzb. Akad. wiss. Wien*, xcix, 1890, 3, p. 110; *Zeit. wiss. Mik.*, viii, 1891, 227). These methods give a differential stain of rods and cones.

852. Dissociation.—For maceration preparations you may use weak solutions (0·2 to 0·5 per cent.) of osmic acid for fixation, and then macerate in 0·02 per cent. chromic acid (M. SCHULTZE), or in iodised serum (M. SCHULTZE), or in dilute alcohol (LANDOLT), or in Müller's solution, or (RANVIER, *Traité*, p. 957) in pure water, for two or three days. THIN (*Journ. of Anat.*, 1879, p. 139) obtained very good results by fixing for thirty-six to forty-eight hours in one third alcohol, or in 25 per cent. alcohol, and then staining and teasing.

SCHIEFFERDECKER macerates fresh retina for several days in the methyl mixture, § 551.

KRAUSE (*Intern. Monatssch. Anat.*, 1884, p. 225) recommends treatment for several days with 10 per cent. chloral hydrate solution; the rods and cones are well preserved.

Inner Ear.

853. Inner Ear, Dissection.—For the dissection of the human ear see POLITZER, "Die anatomische u. histologische Zergliederung d. menschlichen Gehörorganes," Stuttgart (Enke), 1889 (*Zeit. wiss. Mik.*, vii, 1890, p. 364). Amongst the lower mammalia, the *guinea-pig* is a favourable subject, as here (as with some other rodents) the cochlea projects freely into the cavity of the bulla, and may be easily removed with a scalpel and brought into a fixing liquid, and opened therein. With fishes and amphibia also the membranous labyrinth may easily be got away.

854. Preparation.—SCHWALBE (*Beitr. z. Phys.*, 1887; *Zeit. wiss. Mik.*, iv, 1887, p. 90). Fix (cochlea of guinea-pig) for eight to ten hours in "Flemming," wash in water, decalcify (twenty-four hours is enough) in 1 per cent. hydrochloric acid, wash the acid out, dehydrate, and imbed in paraffin. See also FERRERI, § 585 (phloroglucin).

PRENANT (*Intern. Monatsschr. Anat.*, ix, 1892, p. 28).—For sections, open the cochlea in solution of Flemming or of Hermann, and fix therein for four to five hours. Avoid decalcification as far as possible, as it is inimical to good preservation of elements; but if necessary take 1 per cent. palladium chloride. Make paraffin sections.

Isolation preparations of the stria vascularis may be made by putting a cochlea for a day into 1 per cent. solution of osmic acid, then for four to five days into 0·1 per cent. solution; the stria may then be got away whole.

Stein (*Anat. Anz.*, xvii, 1900, p. 398) decalcifies in celloidin by the method of Rousseau; see § 567.

For *staining*, Ranvier (*Traité*, p. 991) employs his gold and formic-acid method, § 374.

The bichromate-and-silver method of Golgi may be employed with fœtal or new-born subjects. The methylen-blue *intra vitam* method has given good results. For the higher vertebrates the injection method should be employed; for fishes and amphibia the immersion method will suffice.

855. Other Methods.—Waldeyer, Stricker's *Handb.*, p. 958 (decalcification either in 0·001 per cent. palladium chloride containing 10 per cent. of HCl, or in chromic acid of 0·25 to 1 per cent.).

Urban Pritchard (*Journ. Roy. Mic. Soc.*, 1876, p. 211).—Decalcification in 1 per cent. nitric acid.

Lavdowsky (*Arch. mik. Anat.*, 1876, p. 497).—Fresh tissues (from the cochlea) are treated with 1 per cent. solution of silver nitrate, then washed for ten minutes in water containing a few drops of 0·5 or 1 per cent. osmic acid solution, and mounted in glycerin.

Max Flesch (*Arch. mik. Anat.*, 1878, p. 300).

Tafani (*Arch. Ital. de Biol.*, vi, p. 207).

Eichler, *Abh. math-phys. Cl. Sächs. Ges. Wiss.*, xviii, 1892, p. 311; *Zeit. wiss. Mik.*, ix, 1893, p. 380 (detailed account of manipulations for injection of blood-vessels of the labyrinth); Siebenmann, *Die Blutgefässe im Labyrinthe des menschlichen Ohres*, Wiesbaden, Bergmann, 1894; *Zeit. wiss. Mik.*, xi, 1894, p. 386.

856. Olfactive Nerve-endings, Tactile Corpuscles, etc.—Besides the *gold method*, Chapter XVII, and the *methylen-blue method*, Chapter XVI, the rapid *bichromate-and-silver method* of Golgi should be employed, and for the olfactive mucosa gives the best results; see van Gehuchten, *op. cit.*, § 813. For *intra-epidermic nerve-endings*, besides the methods given, Chapter XXVII, the Golgi method should be employed. According to van Gehuchten (*La Cellule*, ix, 1893, p. 319) it gives much better results than gold methods. He uses the rapid process. For *tactile corpuscles*, etc., besides the methods given pp. 360 and 361, see § 771.

Electric Organs.

857. Torpedo.—Ballowitz (*Arch. mik. Anat.*, xlii, 1893, p. 460) gets the best results by the Golgi impregnation, controlled by treatment of fresh tissues for one or two days with osmic acid of 1 per cent. and teasing. He finds gold methods and the simple silver nitrate method not good. See further on the whole subject, BALLOWITZ, *op. cit.*, and *Encycl. mik. Technik.*, p. 194.

IWANZOFF (*Bull. Soc. Nat. Moscou*, 2, viii, 1895, p. 407) injects osmic acid of 0·5 to 2 per cent., removes the pillars after a few minutes, and hardens in 2 per cent. bichromate of potash, stains in hæmatoxylin, and imbeds in paraffin.

858. Raja.—IWANZOFF (*op. cit.*, ix, 1895, p. 74) fixes the organ in the tail of *Raja* with liquid of Flemming, stains, and cuts as above.

BALLOWITZ (*Anat. Hefte*, 1 Abth., vii, 1897, p. 285) finds the method of Golgi excellent for this organ. He also makes sections after fixing in saturated solution of sublimate (in sea-water), or in liquid of Flemming, and examines them in water. Methylen-blue may be used, *intra vitam.*

858a. Gymnotus.—BALLOWITZ (*Encycl. mik. Technik.*, p. 201) fixes with *Flemming* or concentrated sublimate, and makes sections. He also commends impregnation with gold chloride, but not the Golgi method.

859. Malapterurus.—BALLOWITZ (*ibid.*, p. 202) fixes with picro-sublimate, with Flemming, or with various mixtures of bichromate, sublimate, and formol, and uses gold chloride and Golgi impregnations. He macerates in liquid of Müller or saturated aqueous solution of picric acid.

Other Motor Nerve-endings.

See Chapters XVI, XVII, XXVII, XXVIII, and XXIX.

CHAPTER XXXVI.

METHODS FOR INVERTEBRATES.

860. Introduction.—The following methods are all of them such as give results applicable to *histological* study, and no account has been taken of such methods as are merely useful for the preparation of organisms for museum specimens or for coarse dissection.

See p. 74 as to the employment of formaldehyde as a preservative.

A paper giving an account of the processes employed in the Naples Zoological Station for the preservation of marine animals has been published by SALVATORE LO BIANCO in *Mitth. Zool. Stat. Neapel*, ix, 1890, p. 435. *References to the works of* S. LO BIANCO *in the remainder of this chapter are to that paper.* An abstract of it is contained in *Amer. Natural*, xxiv, 1890, p. 856, and *Journ. Roy. Mic. Soc.*, 1891, p. 133, and a very full account in *Zeit. wiss. Mik.*, viii, 1891, p. 54

Tunicata.

861. Fixation of Tunicata.—A method of LO BIANCO for killing simple Ascidians in an extended state has been given, § 22. Some forms, such as *Clavellina, Perophora, Phallusia, Molgula, Cynthia,* etc., should first be narcotised by treatment for from three to twelve hours with choral hydrate (1 : 1000 in sea water), then killed in a mixture containing chromic acid of 1 per cent. 10 parts, acetic acid 100 parts, and finally hardened in 1 per cent. chromic acid.

The compound Ascidians with contractile zooids may be treated by the following process (due to VAN BENEDEN, kindly communicated to me by Dr. C. Maurice). Place the corms in clean sea water, and leave them alone for a few hours, in order that the zooids may become fully extended, then plunge them suddenly into glacial acetic acid. Leave them there for two, four, or six minutes, according to the size of the corms (which are best taken of as small a size as possible). Take them out of the acid with your fingers (or at all events not with steel instruments, which would blacken the tissues) and bring them into 50 per cent. alcohol. Wash them thoroughly in that, and then bring them in the usual way through successively stronger alcohols.

I strongly recommend this process, which gives admirably preserved preparations quite free from any opacity either in the tissues or the tunic. The acid will not hurt the fingers if they be washed immediately.

S. LO BIANCO recommends for this group the chloral hydrate process, followed by fixation with sublimate or chromo-acetic acid.

CAULLERY (*Bull. Sc. France Belg.*, xxvii, 1895, p. 5) first stupefies the animals with cocaine (LAHILLE, a few drops of 5 per cent. solution to 30 c.c. of sea water), then fixes in liquid of Flemming or acetic acid.

Most small *pelagic* Tunicates are very easily fixed with osmic acid or acid sublimate solution.

I have found the acetic acid process very good for *Pyrosoma.* LO BIANCO puts them for a quarter of an hour into 50 per cent. alcohol containing 5 per cent. of hydrochloric acid, then into successive alcohols, beginning with 60 per cent. He kills the hard forms of *Salpa* with acetic acid of 10 per cent., the semi-hard ones with 1 per cent. chromic acid containing 5 per cent. acetic acid, the soft ones with 1 per cent. chromic acid containing $\frac{1}{50}$ per cent. osmic acid, or (*Grundzüge*, p. 66) 10 parts of 1 per cent. chromic acid, with 1 of formol and 9 of sea-water, Doliolidæ with sublimate, or the above osmic mixture, or a mixture of 10 parts 10 per cent. solution of sulphate of copper with 1 part concentrated sublimate solution, or the formol mixture.

Molluscoida.

862. Bryozoa.—For some methods of killing and fixing see §§ 11, 18, and 19. S. LO BIANCO employs for *Pedicellina* and *Loxosoma* the chloral hydrate method, fixing with sublimate. For *Flustra, Cellepora, Bugula, Zoobothrium,* he employs the alcohol method of EISIG, § 16. For *Cristatella* see §§ 17, 18.

CONSER (*Trans. Amer. Mic. Soc.*, xvii, 1896, p. 310) kills the fresh-water forms with cocaine, puts them for an hour into 1 per cent. chromic acid, and passes through water into alcohol, etc.

Calvet (*Hist. Nat. Bryozoaires*, Montpellier, 1900, p. 15) fixes marine forms with a mixture of 100 parts 0·3 per cent. chromic acid, with 1 of 3 per cent. hydrochloric acid or 100 of 0·2 per cent. chromic acid with 1 of 5 per cent. hydrochloric. For impregnating with silver he rejects the process of Harmer, and treats with vapour of osmium and washes thoroughly.

863. Brachiopoda.—Lo Bianco kills small animals in 70 per cent. alcohol, larger ones in the same, but after first narcotising with alcohol and sea water.

Blochmann (*Untersuch. fein. Bau Brachiopoden*, Jena, 1892, p. 5) fixes principally with sublimate, macerates by the Hertwigs' method, § 543, decalcifies with 1 per cent. chromic acid (for thick shells add a little hydrochloric or nitric acid), or with nitric acid in alcohol of 50 to 70 per cent., and imbeds in paraffin or celloidin, the latter giving the less shrinkage. For injections, Berlin blue, or 2 per cent. blue or red gelatin.

Ekman (*Zeit. wiss. Zool.*, lxii, 1896, p. 172) fixes the peduncle chiefly with liquid of Flemming, and cuts chiefly with the free hand, in liver, seldom in paraffin.

Mollusca.

864. Fixation of Mollusca.—Two groups at least amongst the Mollusca offer considerable difficulties in the way of fixation—Lamellibranchiata and Gastropoda.

Lo Bianco narcotises Lamellibranchs for six to ten hours or more with alcohol, § 16, and then kills them.

Carazzi (*Mitth. Zool. Stat. Neapel*, xii, 1896, p. 388) employs for *Ostrea* the alcohol method, but warms the vessel containing the animals to about 25° C., and thus obtains total narcosis in twenty-four hours. For the study of the branchiæ he then excises all four lamellæ, fixes them in a sublimate mixture similar to that of Gilson, § 74, for one or two hours (or the entire body of the animal may be excised and fixed for four to six hours), then passes through iodine-alcohol and absolute alcohol, removes the two outer lamellæ, and imbeds the two inner ones, which have suffered less from the operations. Recently (*Grundzüge*, p. 423) he fixes the narcotised animals with 1 part of formol to 3 or 4 of sea water, which prevents shrinkage.

See also § 20.

The methods recommended for Lamellibranchiata sometimes give good results with Gastropoda.

S. Lo Bianco advises that Prosobranchiata, and, amongst the Heteropoda, Atlantidæ, be narcotised with 70 per cent. alcohol, § 16. Opisthobranchiata ought not to give much trouble, and I recommend sudden killing with liquid of Perényi, or the acetic method, § 861. *Aplysia* may first be narcotised by subcutaneous injection of about 1 c.c. of a 5 to 10 per cent. solution of hydrochlorate of cocaine (Robert, *Bull. Scient. de la France*, etc., 1890, p. 449; *Zeit. wiss. Mik.*, ix, 1892, p. 216), or (Schönlein, *Zeit. Biol.*, xxx, 1893, p. 187) 1 c.c. of 4 per cent. solution of Pelletierin. For Lo Bianco's various methods see the original, p. 467.

For Pteropoda in general, liquid of Perényi. *Creseis* is a difficult form. S. Lo Bianco advises the alcohol method, § 16. For the Gymnosomata he narcotises with 0·1 per cent. chloral hydrate.

For *preservation* it may be noted that for Heteropoda and Pteropoda, formaldehyde (*preceded by due fixation* in a chromic or sublimate solution) is an admirable medium, so far at least as macroscopic appearances are concerned, and for this purpose superior to alcohol.

For terrestrial Gastropods see §§ 20 and 23. Marchi (*Arch. mik. Anat.*, 1867, p. 204) gets rid of the mucus of the integument of Limax, which may be an obstacle to preparation, by putting the living animal into moderately concentrated salt solution, in which it throws off its mucus and dies in a few hours.

Lang (*Anat. Hefte*, 1902, p. 84) puts *Helix* into water with enough chromic acid to make it of a Rhine-wine colour, with an air-tight cover to the vessel, and when the animals are extruded injects into them a quarter to a half of a Pravaz syringe of 1 per cent. cocain, and after five to fifteen minutes dissects and fixes.

Heymans (*Bull. Acad. Belg.*, xxxii, 1896, p. 578) injects ethyl bromide under the skin of Cephalopoda.

Lo Bianco uses for fixing them his chromo-acetic acid No. 1 (§ 43), with a double quantity of acetic acid, for twenty-four hours.

865. Central Nervous System of Pulmonata.—B. de Nabias (*Act. Soc. Linn. Bordeaux*, 1894; *Rech. Hist. centres nerveux des Gastéropodes*, 1894, p. 23) opens the animals and fixes the ganglia for one hour in a mixture of 6 parts glacial acetic acid to 100 of 90 per cent. alcohol, or for fifteen to twenty minutes in 5 per cent. sublimate with 5 per cent. acetic acid. He stains in bulk, with Renaut's hæmatoxylic eosin, § 403, or R. Heidenhain's hæmatoxylin, § 281, or the copper hæmatoxylin of Viallanes, § 878, and imbeds in paraffin. He also stains by the rapid method of Golgi, imbedding, however, the ganglia in celloidin directly after the hardening in osmic acid and bichromate, and treating the sections with the silver (p. 34). He stains with methylen blue by treating the ganglia *in situ* for twelve to twenty-four hours with a 1 per cent. solution.

866. Eyes of Gastropoda (Flemming, *Arch. mik. Anat.*, 1870, p. 441).—To obtain the excision of an exserted eye, make a rapid cut at the base of the peduncle, and throw the organ into very dilute chromic acid, or 4 per cent. bichromate; after a short time it will evaginate, and remain as completely erect as if alive. Harden in 1 per cent. osmic acid, in alcohol, or in bichromate.

Carrière (*Zool. Anz.*, 1886, p. 221) removes the eye, together with a portion of the tentacle, and fixes by exposing it for some minutes to vapour of osmic acid. He depigments sections by very careful treatment with *very dilute eau de Javelle.*

867. Eyes of Cephalopoda and Heteropoda (Grenacher, *Abh. naturf. Ges. Halle-a.-S.*, Bd. xvi; *Zeit. wiss. Mik.*, 1885, p. 244).—Fix (Cephalopod eyes) in picro-sulphuric acid, or in a saturated solution of corrosive sublimate in picro-sulphuric acid (this mixture is especially useful for *Octopus*, *Eledone*, and *Sepia*, but does not succeed with the pelagic forms, such as *Loligo*, *Ommatostrephes*, and *Rossia*). Depigment the specimens with hydrochloric acid (in preference to nitric acid). The mixture § 594 may also be used. The operation of depigmentation may be combined with that of staining; if you stain with borax-carmine and wash out in the last-mentioned liquor, the pigment will be found to be removed quicker than the stain is washed out. The operation may be carried out on sections, but it is better to use portions of retina of 2 to 5 mm. in thickness. Grenacher mounted his preparation in castor oil, see § 462.

Similar methods are recommended by the same author for the eyes of **Heteropoda** (see *Abh. naturf. Ges. Halle-a.-S.*, 1886; *Zeit. wiss. Mik.*, 1886, p. 243).

LENHOSSÉK (*Zeit. wiss. Zool.*, lviii, 1894, p. 636; *Arch. mik. Anat.*, xlvii, 1896, p. 45) applies the method of GOLGI to the eyes of Cephalopods.

Similarly KOPSCH (*Anat. Anz.*, xi, 1895, p. 362), but using formol instead of the osmic acid.

HESSE (*Zeit. wiss. Zool.*, lxviii, 1900, p. 418) fixes eyes of Heteropoda with 1 of formol to 4 of water, and (p. 456) bleaches those of Cephalopoda by the methods of GRENACHER and that of JANDER, § 595.

868. Eyes of Lamellibranchiata.—See PATTEN, *Mitth. Zool. Stat. Neapel*, vi, 1886, p. 733, and RAWITZ, *Jena, Zeit. Naturw.*, xxii, 1888, p. 115, and xxiv, 1890, p. 579 (bleaches with caustic soda); see § 596.

HESSE (*op. cit.*, last §, p. 380) employs the method of JANDER for *Arca*. He fixes the eye of *Pecten* in 10 per cent. formol for five minutes, followed by sublimate or picro-nitric acid.

869. Shell.—Sections of non-decalcified shell are easily obtained by the usual methods of grinding, or, which is often a better plan, by the methods of v. Koch or Ehrenbaum, §§ 190, 191. MOSELEY (*Quart. Journ. Mic. Sci.* [2], xxv, 1885, p. 40) decalcifies with nitric acid of 3 to 4 per cent. and then makes sections. This method serves for the study of the eyes of CHITONIDÆ.

870. Injection of Acephala (FLEMMING, *Arch. mik. Anat.*, 1878, p. 252).—To kill the animals freeze them in a salt-and-ice mixture, and throw them for half an hour into lukewarm water. They will be found dead, and in a fit state for injection. The injection-pipe may now be tied in the heart, and then the entire animal is filled and covered up with plaster of Paris, which serves to occlude cut vessels that it is not possible to tie. As soon as the plaster has hardened the injection may be proceeded with.

See also DEWITZ, *Anleit. zur Anfert. zootom. Präp.*, Berlin, 1886, p. 44 (*Anodonta*) and p. 52 (*Helix*).

871. Maceration Methods for Epithelium. — ENGELMANN (*Pflüger's Arch.*, xxiii, 1880, p. 505) macerates the intestine of *Cyclas* in osmic acid of 0·2 per cent. (after having warmed the animal for a short time to 45° to 50° C.), or in concentrated boracic acid solution.

The Intra-cellular Processes of the Cilia.—The entire intracellular fibre apparatus may be *isolated* by teasing fresh epithelium from the intestine of a Lamellibranch (*e. g. Anodonta*) in either bichromate of potash of 4 per cent., or salt solution of 10 per cent. To get good views of the apparatus *in situ* in the body of the cell, macerate for not more than an hour in concentrated solution of boracic or salicylic acid. Very dilute osmic acid (*e. g.* 0·1 per cent.) gives also good results. The "lateral cells" of the gills are best treated with strong boracic acid solution (five parts cold saturated aqueous solution to one part water).

BELA HALLER'S **Mixture**, see § 548; BROCK'S **Medium**, § 540; MÖBIUS'S **Media**, § 544; the second of these is much recommended by DROST (*Morphol. Jahrb.*, xii, 1866, p. 163) for *Cardium* and *Mya*.

See also PATTEN (*Mitth. Zool. Stat. Neapel*, vi, 1886, p. 736). Sulphuric acid, 40 drops to 50 grammes of water, is here recommended as a valuable macerating and preservative agent. Entire molluscs, without the shell, may be kept in it for months.

BERNARD (*Ann. Sci. Nat.*, ix, 1890, p. 191) macerates the mantle of Prosobranchs in a mixture of one part each of glycerin and acetic acid, two parts each of 90 per cent. alcohol and 0·1 per cent. chromic acid, and forty parts water, which acts in from a quarter of an hour to three hours. He also (pp. 102, 306) uses a weak solution of chloride of ruthenium, especially for nerve-tracts, mucus-cells and cilia. Alcohol material may be macerated in a mixture of one part glycerin, two of acetic acid, and forty of water.

Arthropoda.

872. General Methods for Arthropoda.—As general methods for the study of chitinous structures, the methods worked out by Paul Mayer (see §§ 8, 101, 251, and 252) are excellent. It is, at all events, absolutely necessary, in the preparation of *entire organisms or unopened organs*, that all processes of fixation, washing, and staining should be done with fluids possessing great penetrating power. Hence picric acid combinations should in general be used for fixing, and alcoholic fluids for washing and staining. *Concentrated* picro-sulphuric acid (or picro-nitric) is the most generally useful fixative, and 70 per cent. alcohol is the most useful strength for washing out. *Alcoholic* picro-sulphuric acid may be indicated for fixing in some cases.

But if the animals or organs can first be properly opened, the usual methods may be employed.

873. Crustacea.—Some forms are very satisfactorily fixed with sublimate. Such are the Copepoda and the larvæ of Decapoda. It is sometimes indicated to use the sublimate in *alcoholic* solution. Some Copepoda, however (*Copilia, Sapphirina*), are better preserved by means of weak osmic acid, and so are the Ostracoda. In many cases the osmic acid will produce a sufficient differentiation of the tissues, so that further staining may be dispensed with; so for *Copilia* and *Phyllosoma*. The pyrogallic process (§ 383) may often prove helpful in the study of such forms. GIESBRECHT takes for marine Copepods a concentrated solution of picric acid in sea water, to which a little osmic and acetic acid may be added. For fresh-water forms, ZACHARIAS (*Zool. Anz.*, xxii, 1899, p. 72) takes chromo-acetic acid.

For Ostracoda, MÜLLER (*Fauna u. flora d. Golfes von Neapel*, xxi [Ostracoda], 1894, p. 8) recommends fixing in a mixture of five parts of ether and one of absolute alcohol, followed by 70 per cent. alcohol. WOLTERECK (*Zeit. wiss. Zool.*, lxiv, 1898, p. 601) fixes *Cypris* with picric acid, saturated, 100 parts, sublimate, saturated, 50 parts, and 2½ to 5 of acetic acid.

HERBST (*Arch. Entwickmech.*, ix, 1899, p. 292) finds 1 per cent. formol in sea-water good for Crustacea in general.

Nettovitch (*Arb. z. Inst. Wien*, xiii, 1900, p. 3) fixes *Argulus* with liquid of Tellyesniczky, § 55, warmed to 50° C.

874. Tracheata.—Kenyon (*Tufts. Coll. Stud.*, No. 4, 1896, p. 80) fixes Pauropoda in Carnoy's acetic alcohol and chloroform, § 90, cuts them in two for staining, etc., and imbeds in celloidin followed by paraffin.

Duboscq (*Arch. Zool. Expér.*, vi, 1899, p. 483) fixes Chilopoda in a mixture of equal parts of 1 per cent. chromic acid, 10 per cent. nitric acid, and 95 per cent. alcohol, or in a mixture of one part of glacial acetic acid and ten of absolute alcohol.

Hennings (*Zeit. wiss. Mik.*, xvii, 1900, p. 311) takes—Nitric acid 16 parts, chromic acid of 0·5 per cent. 16 parts, sublimate saturated in 60 per cent. alcohol 24 parts, picric acid saturated in water 12, and absolute alcohol 42, fixes for twelve to twenty-four hours, and washes out with iodine alcohol. He says that this mixture not only fixes but softens chitin enough to allow of paraffin sections being made through hard parts.

Concerning both these mixtures *cf.* §§ 41, 42, 52.

Hamann (*Sitz. Naturw. Freunde Berlin*, 1897, p. 2) fixes small Tracheata in 10 per cent. formol, and finds the chitin sufficiently soft for sections to be made.

875. Methods for Clearing and Softening Chitin.—The methods of Looss have been described § 566, those of Hennings and Hamann last §.

List (*Zeit. wiss. Mik.*, 1886, p. 212) has obtained good results with Coccidæ by treating them (after hardening) for eighteen to twenty-four hours with *eau de Javelle*, diluted with four volumes of water. After washing out they may be imbedded in paraffin, the chitin being sufficiently softened to allow of their being penetrated and good sections being obtained.

Sazepin's method for antennæ of Chilognatha (*Mém. Acad. Imp. St. Pétersb.*, xxxii, 1884, pp. 11, 12) consists in steeping antennæ (that have been dehydrated with alcohol) for twenty-four hours in chloroform containing a drop of fuming nitric acid (shake occasionally).

Bethe's method (*Zool. Jahrb.*, viii, 1895, p. 544) for the preparation of *telsons* of *Mysis* is as follows: They are put for eight to fourteen days into 40 per cent. alcohol, to which nitric acid is gradually added, so that by the end of that time they have been brought into alcohol containing 20 per cent. of the acid. This softens the chitin, and somewhat breaks down the structure of the otolith, so that good sections through it are occasionally obtained.

See also the depigmentation processes, §§ 587—596.

876. Test for Chitin (ZANDER, *Pflüger's Arch.*, lxvi, 1897, p. 545). The object is placed in water under a cover-glass, and treated for a short time with a drop of freshly prepared solution of iodine in iodide of potassium. This is then partly removed with water, and a drop of concentrated chloride of zinc added. This is in its turn removed with water as far as possible, and the violet reaction is obtained.

877. BETHE'S **Stain for Chitin** (*loc. cit.*, § 875).—Sections are put for three or four minutes into a freshly prepared 10 per cent. solution of anilin hydrochloride, to which has been added one drop of hydrochloric acid for every 10 c.c. They are then rinsed in water, and the slide is put with the sections downwards into 10 per cent. solution of bichromate of potash. The stain is at first green, but becomes blue in tap water or alcohol containing ammonia.

MAYER (*Grundzüge*, p. 430) simply uses a solution of pyrogallol in alcohol or glycerin.

878. Eyes of Arthropods.—For the methods of LANKESTER and BOURNE (*Quart. Journ. Mic. Sci.*, 1883, p. 180 : *Limulus*) ; HICKSON (*ibid.*, 1885, p. 243 : *Musca*) ; PARKER (*Bull. Mus. Harvard Coll.*, xx, 1890, p. 1 ; *Zeit. wiss. Mik.*, viii, 1891, p. 82 : *Homarus*) see *early editions*.

In a later paper (*Mitth. Zool. Stat. Neapel*, xii, 1895, p. 1) PARKER describes the application of the methylen blue method to the study of the retina and optic ganglia in Decapods, especially in *Astacus*. He injected 0·1 c.c. of a 0·2 per cent. solution into the ventral sinus. After twelve to fifteen hours the animals were killed, the ganglia quickly dissected out, and the stain fixed as described, § 352.

For his method for eyes of *Scorpions* see § 595.

For the methods of PURCELL for the eyes of *Phalangida* see *Zeit. wiss. Zool.*, lviii, 1894, p. 1. He has the following stain. The cephalothorax is removed and brought for twenty minutes into 50 per cent. alcohol warmed to 45° or 50° C., and saturated with picric acid. The pigment dissolves in this solution and stains the nuclei and some other parts of the rhabdoms, so that no further stain is required.

Rosenstadt (*Arch. mik. Anat.*, xlvii, 1896, p. 748) fixes eyes of Decapods in a warm mixture of three parts concentrated sublimate solution and one part liquid of Perényi, and depigmentates them in a mixture of three parts each of nitric and hydrochloric acid and 100 of water, warmed to 56° C. for a few hours.

Viallanes (*Ann. Sci. Nat.*, xiii, 1892, p. 354) fixes eyes of *Palinurus* in 5 per cent. sublimate with 5 per cent. acetic acid, washes out in 70 per cent. alcohol, depigmentates in a mixture of equal parts of alcohol, glycerin, and water, through which chlorine gas is led, puts for twelve hours into 1 per cent. solution of cupric sulphate, washes for five to six hours in distilled water, and stains for twelve hours in a fresh solution of one part hæmatoxylin in 100 of absolute alcohol and 300 of distilled water. He then puts them back for the same time into the copper solution, washes, and passes through alcohol and makes paraffin sections. The sections may be afterwards stained with safranin.

Hennings (*Zeit. wiss. Mik.*, xvii, 1900, p. 326) depigments sections by putting them for ten minutes (Musca) to twelve hours (Myriopoda) into a mixture of 2 parts of 80 per cent. alcohol with one of glycerin and 2 per cent. of nitric acid, best kept at 35° C. The elements are well preserved.

879. Brain of Bees.—Kenyon (*Journ. Comp. Neurol.*, vi, 1896, p. 137; *Journ. Roy. Mic. Soc.*, 1897, p. 80) treats by the Golgi process (seldom successful), or hardens in a mixture of one part formol and two of 5 per cent. sulphate of copper, followed by staining in Mallory's phospho-molybdic hæmatoxylin, § 286.

880. Ventral Cord.—Binet (*Journ. de l' Anat. et de la Phys.*, xxx, 1894, p. 469) fixes the ganglia of Hexapods either in liquid of Flemming, or in Viallanes's sublimate, § 878, treats them with his copper hæmatoxylin, § 878, and makes paraffin sections, which he stains with safranin.

881. Injections (Arachnida and Crustacea especially).—Aimé Schneider (*Tablettes Zool.*, ii, 1892, p. 123) recommends lithographic Indian ink, the animals being narcotised with chloroform, then injected and thrown into strong alcohol. Similarly Causard (*Bull. Sc. France Belg.*, xxix, 1896, p. 16).

882. Arctiscoida (Doyere, *Arch. mik. Anat.*, 1865, p. 105).—Examination of living animals after partial asphyxiation in boiled water. See *early editions.*

Vermes.

883. Enteropneusta.—Lo Bianco (*op. cit.*, p. 460) fixes with picro-sulphuric acid or 0·5 per cent. chromic acid, with previous narcotisation with alcohol if desired.

Willey (*Zool. Results*, etc., iii, 1899, p. 325) takes 100 parts 1 per cent. chromic acid with 2 of 1 per cent. osmic acid, for 12 hours.

884. Myzostoma.—Wheeler (*Mitth. Zool. Stat. Neapel*, xii, 1896, p. 227) fixes with sublimate or picro-acetic acid.

885. Chætopoda: Cleansing Intestine.—Kükenthal (*Journ. Roy. Mic. Soc.*, 1888, p. 1044) puts *Lumbricus* into a tall glass vessel which has been filled up with bits of moistened blotting-paper. They gradually evacuate the earthy particles from the gut, and fill it instead with paper.

Vogt and Yung (*Traité d' Anat. Comp. Prat.*, v) recommend coffee-grounds instead of paper; paper becomes rather hard when imbedded, whereas coffee-grounds cut fairly well.

Joest (*Arch. Entwicklungsmech.* v., 1897, p. 425) simply keeps the worms for a few days in moist linen, and finds the gut empty.

886. Chætopoda: Fixation.—*Lumbricus* may be anæsthetised by putting the animals into water with a few drops of chloroform. Perrier prefers not to let the chloroform act directly in solution on the animals, but to put them into water in a shallow dish, set up a watch-glass with chloroform in the corner of it, and cover the whole. In half an hour the worms will be more or less narcotised, and if allowed to remain will die in a state of extension.

Cerfontaine (*Arch. de Biol.*, x, 1890, p. 327) recommends curare administered by interstitial injection of a dose of about 2 c.c. of a 1 : 500 solution. The animal should afterwards be put into water, and after a quarter of an hour will be found dead.

Jaquet (*Bib. Anat.*, iii, 1895, p. 32) kills *Lumbricus* in extension in 1 part of nitric acid to 125 of water.

Collin (*Zeit. wiss. Zool.*, xlvi, 1888, p. 474) puts *Criodrilus lacuum* into a closed vessel with a little water, and hangs up in it a strip of blotting-paper soaked in chloroform. Kükenthal (*Die mik. Technik*, 1885; *Zeit. wiss. Mik.*, 1886, p. 61) puts Annelids into a glass cylinder filled with water to the height of 10 centimetres, and then pours 70 per cent. alcohol to a depth of one to two centimetres on to the water. The animals will be found sufficiently narcotised for fixation in from four to eight hours. For Opheliadæ he also employs 0·1 per cent. of chloral hydrate in sea water.

Many marine Chætopoda may be successfully narcotised (S. Lo Bianco) in sea water containing 5 per cent. of alcohol, or by means of the mixture § 16.

The *Polychæta sedentaria* offer the difficulty of a complex and very contractile branchial apparatus. They may sometimes be satisfactorily fixed by bringing them rapidly into corrosive sublimate. Cold, not hot, solutions should be taken, as heat frequently shrivels up the branchiæ. The species of *Polychæta errantia* that offer a contractile branchial apparatus, as *Eunice* and *Onuphis*, may be treated in the same way.

S. Lo Bianco advises killing Chætopteridæ, Sternaspidæ, *Spirographis*, *Protula*, by putting them for half an hour into 1 per cent. chromic acid. I have satisfied myself that good show specimens can be obtained in this way; but I doubt the histological preservation of the parts being so good as with sublimate specimens. Some of the *sedentaria* may be got protruded from their tubes by leaving them for some hours in 0·1 per cent. chloral hydrate in sea water (S. Lo Bianco).

Rievel (*Zeit. wiss. Zool.*, lxii, 1896, p. 292) fixes *Ophryotrocha* in extension in hot liquid of Lang (§ 71), five to eight minutes, and *Lumbricus* in hot alcoholic sublimate or hot picro-sulphuric acid, ten to fifteen minutes.

For Eisig's methods for Capitellidæ see *Fauna u. Flora Golf. Neapel*, xvi, 1887, p. 295, or *Grundzüge*, p. 432.

See also § 12 (lemon juice), and the methods §§ 18 to 24, 43, and 53.

887. Staining.—For the staining of small Annelids entire I find carmalum gives very good results, I think better than borax-carmine or paracarmine.

888. Blood-vessels of Annelids (Kükenthal, *Zeit. wiss. Mik.*, 1886, p. 61).—The animals should be laid open and put for two or three hours into *aqua regia* (4 parts of nitric acid to 2 of hydrochloric acid). The ramifications of the vessels will then be found to be stained black, the rest of the preparation yellow.

Bergh (*Anat. Hefte*, xlv, 1900, p. 392, and xlix, 1900, p. 599) puts small Annelids for a week or more into equal parts of 1 per cent. nitric acid and 1 per cent. nitrate of silver, or into 50 parts of nitrate, 25 of formic acid, and 25 of water, dissects out the organs and exposes to light. Intra-vascular injection is not so good. Marine forms may be treated by Harmer's process (§ 365).

889. Nerves of Annelids.—The methylen blue method and the bichromate of silver method of Golgi (the *rapid* method). For the latter see v. Lenhossék (*Arch. mik. Anat.*, xxxix, p. 102).

See also M. Lewis, *Anat. Anz.*, xii, 1896, p. 292; Atheson, *ibid.*, xvi, 1899, p. 497; and the methods of Apáthy, §§ 350, 377, 380, and 774.

890. Hirudinea.—For the methods of killing see those given for *Lumbricus* in § 886, also §§ 18 to 24, and 53.

Whitman (*Meth. in mic. Anat.*, p. 27) recommends that they be killed with sublimate.

I have obtained better results myself by narcotising with carbonic acid (§ 24), and fixing with liquid of Flemming. I have also found that *lemon juice* kills them in a state of very fair extension. Carmalum I find excellent for staining entire animals. Ehrlich-Biondi mixture sometimes gives fine results with sections.

Graf (*Jen. Zeit.*, 1893, p. 165) states that he has obtained good results by narcotising with decoction of tobacco.

Injection.—Whitman (*Amer. Natural.*, 1886, p. 318) states that very perfect natural injections may often be obtained from leeches that have been hardened in weak chromic acid or other chromic liquid. He considers that these injections are the best for the purpose of the study of the circulatory system by means of sections.

Jacquet (*Mitth. Zool. Stat. Neapel,* 1885, p. 298), for artificial injections, puts leeches into water with a very small quantity of chloroform; they soon fall to the bottom of the vessel and remain motionless. They should be allowed to remain a day or two in the water before injecting them.

Nervous system.—Impregnation with gold. Bristol (*Journ. of Morph.,* xv, 1898, p. 17) kills in formic acid of 15 to 20 per cent., puts for twenty-five minutes into 1 per cent. gold chloride, reduces in formic acid of 1 per cent. (twelve to eighteen hours), and imbeds in paraffin. See also §§ 350, 377, 380, and 774.

891. Gephyrea.—Vogt and Yung (*Anat. Comp. Prat.,* p. 373) direct that *Siphunculus nudus* be kept for some days in perfectly clean basins of sea water, changed every day, in order that the intestine of the animals may be got free from sand, and then anæsthetised with chloroform, under which treatment they die extended.

Ward (*Bull. Mus. Comp. Zool., Cambridge, Harvard Coll.,* xxi, 3, p. 144) puts them into a shallow dish with sea water and pours 5 per cent. alcohol in a thin film on to the surface of the water. After four to eight hours, if the animals make no contractions on being stimulated, they may be removed to 50 per cent. alcohol.

S. Lo Bianco says killing with 0·5 per cent. chromic acid or with 0·1 per cent. chloral hydrate in sea water may be tried, but either method is uncertain. *Phascolosoma* and *Phoronis* should be treated by the alcohol method.

Apel (*Zeit. wiss. Zool.,* xlii, 1885, p. 461) says that *Priapulus* and *Halicryptus* can only be satisfactorily killed by heat. The animals may either be put into a vessel with sea water and be heated on a water-bath to 40° C.; or they may be thrown as rapidly as possible into boiling water, which paralyses them so that they can be quickly cut open and thrown into $\frac{1}{3}$ per cent. chromic acid or picro-sulphuric acid.

892. Rotatoria.—For quieting them for study in the living state, WEBER (*Arch. de Biol.*, viii, 4, 1888, p. 713) finds that 2 per cent. solution of hydrochlorate of cocaine gives the best results. Warm water gave him good results for large species, such as those of *Hydatina* and *Brachionus*.

HARDY (*Journ. Roy. Mic. Soc.*, 1889, p. 475) recommends thick syrup added drop by drop to the water. HUDSON (*ibid.*, p. 476) mentions weak solution of salicylic acid.

See also §§ 20, 21, 25, and 917. Methylen blue, § 347, may be found useful.

Permanent preparations may be made by the method of ROUSSELET (*Journ. Quekett Mic. Club*, v, March, 1895, p. 1): The animals are got together in a watch glass and are narcotised by adding to the water at intervals a few drops of the following mixture:

Hydrochlorate of cocaine 2 per cent. solution	3 parts.
Methylated spirit	1 part.
Water	6 parts.

They are watched under a dissecting microscope, and at the moment when the cilia have ceased to beat, or are seen to be on the point of ceasing to beat, they are fixed by adding a drop of liquid of Flemming or of $\frac{1}{4}$ per cent. osmic acid. This is allowed to act for half a minute or less, after which the animals are taken out with a pipette, and thoroughly washed by passing them through two or three watch glasses of distilled water. They are then definitely mounted in $2\frac{1}{2}$ per cent. solution of formaldehyde (formol $2\frac{1}{2}$ parts, distilled water $37\frac{1}{2}$ parts).

ZOGRAF (*Comptes Rend.*, cxxiv, 1897, p. 245) narcotises as ROUSSELET, but without the spirit, fixes with osmic acid for two to four minutes, then replaces this by raw pyroligneous acid diluted with eight to ten volumes of water, and after five to ten minutes washes in several changes of water, and passes through successive alcohols into glycerin or balsam.

CONSER (*Trans. Amer. Mic. Soc.*, xvii, 1896, p. 310) narcotises with cocaine, and fixes with 20 per cent. formol followed by 0·5 per cent. chromic acid.

LENSSEN (*La Cellule*, xiv, 1898, p. 428) for the embryology of *Hydatina*, kills with hot saturated sublimate, dehydrates, stains lightly, imbeds in paraffin, and stains with hæmalum.

893. Acanthocephali.—SAEFFTIGEN (*Morphol. Jahrb.*, x, 1884,

120) obtained the best results by killing gradually with 0·1 per cent. osmic acid; the animals placed in this contract during the first hours, but stretch out again and die fully extended.

Another method of killing is treatment with 0·1 per cent. chromic acid; Echinorhynci live for days in it, but eventually die fully extended.

Hamann (*Jen. Zeit.*, xxv, 1890, p. 113) has succeeded with sublimate, and also with alcohol containing a little platinum chloride.

Kaiser (*Biblioth. Zool.*, H. vii, 1 Hälfte, 1891, p. 3) found that a saturated aqueous solution of cyanide of mercury, warmed to 45° to 50° C., and allowed to act for from fifteen to sixty minutes, and then washed out with 70 per cent. alcohol, was the best of all fixing media.

He also found the following mixture excellent:

Picric acid	1 gramme.
Conc. sulphuric acid	10 grammes.
Chromic acid	1 gramme.
Water	1000 grammes.

To be warmed to 55° C., allowed to act for fifteen to twenty minutes, washed out for five to ten minutes with hot water, and afterwards for some days in 60 per cent. alcohol.

894. Nematodes.—The extremely impermeable cuticle of these animals is a great obstacle to preparation. According to Looss (*Zool. Anz.*, 1885, p. 318) this difficulty may be overcome in the manner described in § 566.

For fixing, most recent authors recommend sublimate solutions; chromic solutions seem to have a tendency to make the worms brittle.

But, according to Zur Strassen (*Zeit. wiss. Zool.*, liv, p. 655), *Bradynema rigidum* ought to be fixed for at least twelve hours in mixture of Flemming.

Augstein (*Arch. Naturg.*, lx, 1894, p. 255) found that for *Strongylus filaria* the best fixing agent was Mayer's picro-nitric acid.

Vejdovsky (*Zeit. wiss. Zool.*, lvii, 1894, p. 645) advises for *Gordius* 0·5 per cent. chromic acid (twenty-four hours).

Lo Bianco (*loc. cit.*, p. 462) employs for marine forms concentrated sublimate or picro-sulphuric acid.

Looss (*Zool. Anz.*, xxiv, 1901, p. 309) prefers hot (80° to 90° C.) alcohol of 70 per cent.

Cobb (*ante*, p. 3) uses his differentiator for bringing through the various media after fixation.

Staining is frequently difficult, and sometimes alcoholic carmine, § 251, is the only thing that will give fair results.

Braun (see *Journ. Roy. Mic. Soc.*, 1885, p. 897) recommends that small unstained Nematodes be mounted in a mixture of 20 parts gelatin, 100 parts glycerin, 120 parts water, and 2 parts carbolic acid, which is melted at the moment of using. Canada balsam, curiously enough, is said to sometimes make Nematodes opaque.

Demonstration of living Trichinæ (Barnes, *Amer. Mon. Mic. Journ.*, xiv, 1893, p. 104; *Journ. Roy. Mic. Soc.*, 1893, p. 406).—A piece of trichinised muscle of the size of a pea should be placed in a bottle in a mixture of 3 gr. of pepsin, 2 dr. of water, and 2 minims of hydrochloric acid. The whole should be kept at body temperature for about three hours with occasional shaking. The flesh and cysts being dissolved, the fluid is poured into a conical glass, and allowed to settle; the trichinæ are drawn off from the bottom with a pipette, got on to a slide with water, and examined on a hot stage.

Graham (*Arch. mik. Anat.*, l, 1897, p. 216) isolates Trichinæ by macerating for one or two days in 2 per cent. acetic acid, staining with aceto-carmine, and teasing.

895. Nemertina.—After considerable experience of this difficult group I have to say that I know of no method of fixation that will certainly give good results. My best results have always been obtained with cold saturated sublimate solution, acidified with acetic acid. I have tried most of the other usual fixing agents, such as the osmic and chromic mixtures, and do not recommend them for this group, for they seem (the chromic mixtures and perchloride of iron in particular) to act as irritants, and provoke such violent muscular contractions that the whole of the tissues are crushed out of shape by them. And, besides, they do not kill as quickly as sublimate.

Prof. du Plessis has suggested to me fixing with hot (almost boiling) water. On the few occasions on which I have tried it the animals have died in extension, without vomiting their proboscis; and I think it is worth trial, especially for the larger forms.

I have tried Foettinger's chloral hydrate method (§ 18).

My specimens died fairly extended, but vomited their proboscides. According to S. Lo Bianco (*loc. cit.*, p. 461) narcotisation with a solution of 0·1 to 0·2 per cent. in sea water for six to twelve hours is useful.

Oestergren (§ 16) recommends his ether water.

Dendy (see *Journ. Roy. Mic. Soc.*, 1893, p. 116) has succeeded with *Geonemertes* by exposing it for half a minute to the vapour of chloroform.

For staining fixed specimens *in toto* I hold that it is well-nigh necessary to employ alcoholic stains, for even the most delicate species are not satisfactorily penetrated by watery stains in any reasonable lapse of time. Borax-carmine or Mayer's alcoholic carmine may be recommended; not so cochineal or hæmatoxylin stains, on account of the energy with which they are held by the mucin which in general exists in such great abundance in the skin of these animals.

Sections by the paraffin method, after penetration with oil of cedar (chloroform will fail to penetrate sometimes after the lapse of weeks).

Bürger (*Fauna u. Flora Golf. Neapel*, xxii, 1895, p. 443) studies the nervous system, nephridia, skin, muscle, and intestine by the *intra vitam* methylen-blue method. He *injects* the animals with 0·5 per cent. solution in distilled water, or 0·5 per cent. salt water, and allows them to lie for six to twelve hours or more in moist blotting-paper.

He also employs maceration in one third alcohol, or the Hertwigs' medium, § 543.

See also Montgomery ((*Zool. Jahrb. Abth. Morph.*, x, 1897, p. 6 ; and Böhmig (*Zeit. wiss. Zool.*, lxiv, 1898, p. 484).

896. Cestodes.—This group must of course be chiefly studied by the usual section methods. As pointed out by Vogt and Yung (*Traité d'Anat. Comp. Prat.*, p. 204), the observation of the living animal may be of service, especially in the study of the excretory system. And, as shown by Pintner, Tæniæ may be preserved alive for several days in common water to which a little white of egg has been added.

Tower (*Zool. Jahrb.*, xiii, 1899, p. 363) has kept *Moniezia expansa* alive for several days in a mixture of 100 c.c. of tap water, 10 gr. of white of egg, 2 of pepsin, 2 of sugar, and 5 of prepared beef ("Bovox"). Chloride of sodium, he says, should be avoided.

Lönnberg (*Centralb. Bakteriol.*, xi, 1892, p. 89; *Journ. Roy. Mic. Soc.*, 1892, p. 281) has kept *Triænophorus nodulosus*, a parasite of the pike, alive for a month in a slightly acid pepsin-peptone solution containing from 3 to 4 per cent. of nutritive matter and less than 1 per cent. of NaCl.

For the nervous system, Tower (*Zool. Anz.*, xix, 1896, p. 323) fixes in a picro-platin-osmic mixture (stronger than that of O. vom Rath, § 108) for ten hours, then treats for several hours with crude pyroligneous acid, and lastly with alcohol, and imbeds in paraffin.

Zernecke (*Zool. Jahrb., Abth. Anat.*, ix, 1895, p. 92) has employed with success the bichromate of silver impregnation of Golgi. He kills *Ligula* in the osmio-bichromic mixture (4 : 1), impregnates as usual, makes sections in liver, and treats them by the hydroquinon process of Kallius. Besides the peripheral and central nervous system, muscle-fibres, parenchyma cells, and the excretory vascular system are impregnated.

He has also obtained good results by the methylen-blue method.

Blochmann (*Biol. Centralb.*, xv, 1895, p. 14) recommends for the nervous system the bichromate and sublimate method of Golgi.

See also Köhler, *Zeit. wiss. Zool.*, lvii, 1894, p. 386 (stretches Tæniæ round a glass plate or on cork, and fixes with 5 per cent. sublimate).

897. Trematodes (Fischer, *Zeit. wiss. Zool.*, 1884, p. 1).—*Opisthotrema cochleare* may be mounted entire in balsam. For sectioning, Fischer recommends imbedding in a mass made by dissolving 15 parts of soap in 17·5 parts of 96 per cent. alcohol. This mass melts at about 60° C., penetrates very rapidly, and solidifies very quickly. The sections should be studied in glycerin.

Lo Bianco (*loc. cit.*, p. 460) fixes Trematodes with hot saturated sublimate.

Looss (*Arch. mik. Anat.*, 1895, p. 7) takes for *Bilharzia* warm (50° to 60° C.) 1 per cent. sublimate in 70 per cent. alcohol.

Bettendorf (*Zool. Jahrb., Abth. Morph.*, x, 1897, p. 308) has had good results with the rapid Golgi method only on *Distoma hepaticum*, and prefers methylen blue.

Havet (*La Cellule*, xvii, 1900, p. 353) has also had results with the Golgi method on this form, and also with thionin, (after fixing with sublimate), which demonstrates tigroid substance.

Cercariæ.—Schwarze (*Zeit. wiss. Zool.*, xliii, 1886, p. 45) found that the only fixing agent that would preserve the histological detail of these forms was cold saturated sublimate solution warmed to 35°—40° C.

For an "indifferent" liquid, Hofmann (*Zool. Jahrb.*, xii, 1899, p. 176) takes 1 part of white of egg in 9 of normal salt solution.

898. Turbellaria.—For *Rhabdocœla*, Braun (*Zeit. wiss. Mik.*, iii, 1886, p. 398) proceeds as follows: For preparing entire animals, the specimens are got on to a slide, lightly flattened out with a cover, and killed by running under the cover a mixture of three parts of liquid of Lang with one of 1 per cent. osmic acid solution. Other fixing media than that described were not satisfactory. (Böhmig [*ibid.*], commenting on this, says that for some of the tissues, such as muscle and body parenchyma, nitric acid and picro-sulphuric acid are very useful.) Sections may be made by the paraffin method.

Delage (*Arch. de Zool. exp.*, iv, 2, 1886) recommends fixation (of Rhabdocœla Acœla) by an osmium-carmine mixture, for which see *loc. cit.*, or by concentrated solution of sulphate of iron. Liquid of Lang was not successful.

For staining, he recommends either the osmium-carmine or impregnation with gold ($\frac{1}{3}$ formic acid, two minutes; 1 per cent. gold chloride, ten minutes; 2 per cent. formic acid, two or three days in the dark. It is well to allow an excessive reduction to take place, and then lighten the stain by means of 1 per cent. solution of cyanide of potassium).

Böhmig (*Zeit. wiss. Mik.*, iii, 1886, p. 239) says that he has obtained very instructive images with Plagiostomidæ fixed with sublimate and stained with the osmium-carmine.

Graff (*Turbellaria Acœla*, Leipzig, 1891; *Zeit. wiss. Mik.*, ix, 1892, p. 76) says that chromo-aceto-osmic acid, followed by hæmatoxylin, is good for the skin, but will not afford a satisfactory preservation of the Rhabdites, which in Acœla and Alloiocœla seem to be destroyed by swelling. The same method is also good for the parenchyma of *Amphichœrus cinereus, Convoluta paradoxa,* and *C. sordida.* Sublimate is good for *Convoluta Roscoffensis.* For some forms it is important to avoid picro-carmine, which destroys the central parenchyma. The nervous system may be investigated by the methods of DELAGE.

For Dendrocœla sublimate solutions, sometimes hot, appear indicated for fixing. CHICHKOFF (*Arch. de Biol.*, xii, 1892, p. 438) recommends the following for fresh-water Dendrocœla: 2 per cent. sublimate solution, 6 parts; 15 per cent. acetic acid, 4 parts; pure nitric acid, 2 parts; 14 per cent. chloride of sodium, 8 parts; and 2 per cent. alum, 1 part. Note also the mixtures of Lang, § 71. Mayer's tincture of cochineal, § 252, may be found useful for the study of glands, for which purpose the Ehrlich-Biondi stain may also be employed.

OESTERGREN narcotises *Dendrocœlum* with his ether-water, § 16.

LO BIANCO (*loc. cit.*, p. 461) kills Rhabdocœla and Dendrocœla with hot sublimate, throws them at once into cold water, and then brings them into alcohol. For some Polyclads the sublimate must not be more than slightly warm.

VOIGHT (*Verh. Nat. Ver. Bonn*, 1896, p. 118) kills *Planaria* with a mixture of one part concentrated nitric acid and three parts water, and after one minute brings into alcohol of 70 to 90 per cent.

KLINCKOWSTROEM (*Arch. mik. Anat.*, xlviii, 1897, p. 589) fixes *Prostheceræus* in 70 per cent. alcohol with 4 per cent. of acetic acid.

JAENICHEN (*Zeit. wiss. Zool.*, lxii, 1896, p. 256) advises for *Planaria*, eyes especially, picro-sulphuric acid for an hour or two; osmic acid is not good, and liquid of Müller macerates. He stains with borax-carmine, makes sections, and puts them for ten minutes into osmic acid, then for five minutes into pyroligneous acid, on the top of the stove. He macerates the visual rods in a mixture of one part common salt, one of acetic acid, and 100 of water. He bleaches the pigment of the eyes with peroxide of hydrogen.

Echinodermata.

899. Holothurioidea.—These are difficult to fix on account of their contracting with such violence under the influence of irritating reagents as to expel their viscera through the oral or cloacal aperture.

S. Lo Bianco (*loc. cit.*, p. 459) puts them into pure sea water until they have expanded their tentacles, then seizes them with forceps or the fingers behind the tentacles, so as to mechanically render impossible their withdrawal, and immerses the anterior part of the body in acetic acid, whilst at the same time an assistant injects 90 per cent. alcohol through the anus.

Vogt and Yung (*Anat. Comp. Prat.*, p. 641) say that *Cucumaria Planci* (*C. doliolum*, Marenzeller) is free from the vice of expelling its intestines under irritation; but they recommend that it be killed with fresh water, or by slow intoxication with alcohol, chromic acid, or sublimate added to the sea water in which it is contained.

Synapta may be allowed to die in a mixture of equal parts of sea water and ether or chloroform (S. Lo Bianco).

Oestergren (§ 16) puts *Synapta* into his ether-water, but Dendrochirota first into magnesium sulphate of 1 to 2 per cent., for some hours.

Hérouard (*Arch. Zool. Expér.*, vii, 1899, p. 537) kills *Cucumaria* by plunging into a 1 per cent. solution of chloral hydrate warmed to 40° C., the anus being closed by means of forceps.

Gerould (*Bull. Mus. Harvard Coll.*, xxix, 1896, p. 125) paralyses *Caudina* with sulphate of magnesia, § 21, and fixes with liquid of Perényi (or sublimate for the ovaries).

Holothurids, Dr. Weber informs me, are admirably preserved in formaldehyde; a weak solution is sufficient.

For the staining of muscles with methylen blue see Iwanzoff, *Arch. mik. Anat.*, xlix, 1897, p. 103.

900. Echinoidea.—I advise that they be killed by *injection* of some fixing liquid. For preservation, formaldehyde has proved *admirable* in all respects, and greatly superior to alcohol (Weber).

Lo Bianco (*loc. cit.*, p. 458) kills by pouring over them (mouth upwards) a mixture of ten parts acetic acid and one of 1 per cent. chromic acid, and brings at once into weak alcohol. Or he makes two holes in the shell, lets the water run out and alcohol run in.

Sections of spines may be made by grinding, see § 190.

901. Asteroidea.—Hamann (*Beitr. Hist. Echinodermen*, ii, 1885, p. 2) finds it best to *inject* the living animal with a fixing liquid. The cannula should be introduced under the integument at the extremity of a ray, and the liquid injected into the body-cavity. The ambulacral feet and the branchiæ are soon distended by the fluid, and as soon as it seems to have penetrated sufficiently the animal is thrown into a quantity of the same reagent.

In order to study *the eyes*, with the pigment preserved *in situ*, they should be removed by dissection, should be hardened in a mixture of equal parts of 1 per cent. osmic acid and 1 per cent. acetic acid, and sectioned in a glycerin gum mass, or some other mass that does not necessitate treatment with alcohol (which dissolves out the pigment, leaving the pigmented cells perfectly hyaline). For maceration use one third alcohol, the aceto-osmic mixture failing to preserve the rods of the pigmented cells.

Formaldehyde is *not* to be recommended for the preservation of Asteroidea (Weber).

See also Lo Bianco, *loc. cit.*, p. 458 (he kills *Brisinga* with absolute alcohol), also §§ 15, 18.

902. Ophiuridea should in general be killed in fresh water if it be desired to avoid rupture of the rays (De Castellarnau, *La Est. Zool. de Napoles*, p. 135).

Lo Bianco (*loc. cit.*, p. 458) kills small forms with weak alcohol, *Ophiopsila* with absolute alcohol, and *Ophiomyxa* with 0·5 per cent. chromic acid.

Russo (*Ricerche Lab. Anat. Roma*, iv, 1895, p. 157) fixes *Ophiothrix* for an hour or two in 0·5 per cent. osmic acid and then decalcifies in solution of Müller for six to ten days. Or he fixes for three minutes in a mixture of two parts concentrated sublimate solution, one part 70 per cent. alcohol, and one part acetic acid (sp. gr. 1·06), and decalcifies in Müller or in 70 per cent. alcohol with 10 per cent. of acetic acid. He stains with paracarmine.

903. Crinoidea.—Lo Bianco (*loc. cit.*, p. 458) fixes *Antedon rosacea* with 70 per cent. alcohol, *A. phalangium* with 90 per cent.

903a. Larvæ of Echinodermata (from instructions written down for me by Dr. Barrois).—In order to a fruitful study of the metamorphoses of the Echinoidea and Ophiuridea it is necessary to obtain preparations that give distinct images of the different organs, and show the *calcareous skeleton preserved* intact (a point of considerable importance, since this skeleton frequently affords landmarks of the greatest value), and that give clear views of the region of formation of the young Echinoderm (which is generally opaque in the living larva). They should also possess sufficient stiffness to allow of the larva being turned about in any desired way, and placed in any position under the microscope.

Pluteus larvæ should be fixed in a cold saturated solution of corrosive sublimate, in which they remain not more than two or three minutes. They are then washed with water, and brought into dilute Mayer's cochineal (§ 252). This should be so dilute as to possess a barely perceptible tinge of colour. They should remain in it for from twelve to twenty-four hours, being carefully watched the while, and removed from it at the right moment and mounted in balsam, or, which is frequently better, in oil of cloves or cedar-wood.

Auricularia and Bipinnaria —The method described above is equally applicable to these forms, and seems to be altogether the best method for the study of the metamorphosis of Bipinnaria. The earlier stages of the metamorphosis of Auricularia are better studied by fixing with osmic acid, staining with Beale's carmine, and mounting in glycerin.

Larvæ of Comatula are best fixed with liquid of Lang, and stained with dilute borax-carmine. It is important (for preparations that are not destined to be sectioned) to use only *dilute* borax-carmine, as the strong solution produces an over-stain that cannot easily be reduced.

Narcotisation by chloral hydrate before fixing is useful, especially for the study of *Pentacrinus* larvæ and of the young *Synaptæ* formed from Auricularia. Without this precaution you generally get preparations of larvæ either shut up (*Pentacrinus*), or entirely deformed by contraction (young *Synaptæ*).

See also MacBride on the development of *Amphiura squamata*, *Quart. Journ. Mic. Sci.*, xxxiv, 1892, p. 131 (osmic acid followed by liquid of Müller and alcohol; decalcification with nitric acid in alcohol; staining with Mayer's paracarmine or hæmalum); and Seeliger on the development of *Antedon*, *Zool. Jahrb.*, *Abth. Anat.*, vi, 1892, p. 161.

MacBride (*Quart. Journ. Mic. Sci.*, xxxviii, 1896, p. 340) fixes larvæ of *Asterina* in osmic acid, brings into liquid of Müller for twelve to fourteen hours, imbeds in celloidin followed by paraffin (see § 184), and stains sections with carmalum or Delafield's hæmatoxylin, best after a foregoing stain of twenty-four hours in borax carmine.

Cœlenterata.

904. Thread-cells.—Iwanzoff (*Bull. Soc. Nat. Moscou*, x, 1896, p. 97) advises for the Nematocysts of Actiniæ maceration by the Hertwigs' method, § 543, or better, fixation for two to five minutes with vapour of osmium followed by a short washing with sea water or distilled water.

For Medusæ he also advises the Hertwigs' method, § 543, or treatment with a solution containing methyl green and gentian violet with a little osmic acid.

Little (*Journ. App. Mic.*, vi, 1903, p. 2116; *Journ. Roy. Mic. Soc.*, 1903, p. 237) kills *Hydra* in hot saturated sublimate in 70 per cent. alcohol, washes with alcohol, stains for five minutes in strong solution of methylen blue, dehydrates rapidly, clears with cedar or bergamot oil, and mounts in balsam. Nematocytes blue, the rest unstained.

905. Actinida.—*Narcotisation.*—For suitable narcotisation methods see §§ 13 to 23.

Fixation.—In *Le Attinie, Fauna u. Flora d. Golfes v. Neapel*, Andres says that hot corrosive sublimate often gives good results. In the case of the larger forms the solution should be injected into the gastric cavity, and a further quantity of the liquid be poured over the animals.

Freezing sometimes gives good results. A vessel containing Actiniæ is put into a recipient containing an ice-and-salt freezing mixture and surrounded by cotton-wool. After freezing, the block of ice containing the animals is thawed in alcohol or some other fixing liquid.

Duerden (*Journ. Inst. Jamaica*, ii, 1898, p. 449) narcotises with magnesium sulphate, § 21, and fixes with formol of 3 to 5 per cent.

See also Lo Bianco, *loc. cit.*, p. 448.

Maceration.—For the Hertwigs' method (*Jen. Zeit.*, 1879, p. 457) see § 543. The tissues should be left to macerate in the acetic acid for at least a day, and may then be teased in glycerin.

List (*Zeit. wiss. Mik.*, iv, 1887, p. 211) treats tentacles of *Anthea cereus* and *Sagartia parasitica* for ten minutes with a mixture of 100 c.c. of sea water with 30 c.c. of Flemming's strong liquid (§ 47), then washes out for two or three hours in 0·2 per cent. acetic acid, and teases in dilute glycerin. Picro-carmine may be used for staining.

Nervous system.—This group is generally held to be refractory to the Golgi impregnation. Havet, however (*La Cellule*, xviii, 1901, p. 388), has obtained good results by the rapid method on young specimens of *Metridium dianthus* (Ellis). Besides nerve-cells, there are impregnated neuromuscular cells, gland-cells, and nematocytes. Leave for 5 to 8 days in the osmic mixture. He has also had good results by the *intra vitam* methylen blue method (this is also good for nematocytes).

906. Zoantharia with Calcareous Skeletons are difficult to deal with on account of the great contractility of the polyps. Sublimate solution, which ought very often to be taken boiling, sometimes gives good results. De Castellarnau (*La Est. Zool. de Napoles*, p. 132) says that this process succeeds well with *Dendrophyllia*, *Antipathes*, *Astroides*, *Cladocora*, and *Caryophyllia*.

See also Lo Bianco, *loc cit.*, p. 446.

Sections.—Besides the usual methods for sectioning decalcified specimens, see §§ 190 and 191, for undecalcified specimens.

907. The Alcyonaria have also extremely contractile polyps. In a former edition I suggested for their fixation either hot sublimate solution or glacial acetic acid (§ 89). S. Lo Bianco has since recommended essentially similar processes. GARBINI (*Manuale,* p. 151) drenches them with ether, and brings into strong alcohol.

WILSON (*Mitth. Zool. Stat Neapel,* 1884, p. 3) kills Alcyonaria with a mixture of one part of strong acetic acid and two parts of concentrated solution of corrosive sublimate, the animals being removed as soon as dead and hardened for two or three hours in concentrated sublimate solution.

908. Zoantharia and Alcyonaria.—BRAUN (*Zool. Anz.,* 1886, p. 458) inundates *Alcyonium palmatum, Sympodium coralloides, Gorgonia verrucosa, Caryophyllia cyathus,* and *Palythoa axinellæ* with a mixture of 20 to 25 c.c. of concentrated solution of sublimate in sea water with four to five drops of 1 per cent. osmic acid. This is allowed to act for five minutes, and is followed by successive alcohols.

(This method also gives good results with *Hydra* and some Bryozoa and Rotifers.)

See also § 12.

909. Hydroidea, Polypoid Forms.—For suitable *narcotisation* methods see §§ 13 *et seq.*

For killing by *heat* see § 11.

Fixation.—In general the polyps may be very well killed in saturated sublimate solution, in which they should be plunged for an instant merely, and be brought into alcohol. The solution should be employed cold in general for Gymnoblastea, hot for most Calyptoblastea.

Ether attentively administered gives good results with Campanularidæ. *Hydra* is very easily killed by a drop of osmic acid on a slide.

For the *methylen-blue intra vitam* method, see ZOJA, *ante,* p. 228.

910. Medusæ: Fixation.—For narcotisation see § 15. There is some difficulty in properly fixing the forms with contractile tentacles, which easily roll up on contact with reagents. I recommend the following manipulation, due to LO BIANCO. Put sufficient acetic acid into a deepish dish, hold it in your left hand (or, better, in both hands if you have an assistant), and keep it moving in a circle so as to communicate a vortex motion to the liquid. Take up a medusa in a spoon with as little sea water as possible, and throw it into the moving liquid, and keep the liquid steadily swirling round so as to cause the tentacles to trail out at full length behind the animal until it is thoroughly fixed, then pass carefully into alcohol. Do not, unless you are very expert, try to fix more than one medusa at a time ; it is also better to keep the specimens separate, even in the alcohol, as, if several are together, it generally happens that their tentacles become entangled. *Oceania conica* and *Tiara* may usefully, according to Lo Bianco, be narcotised with 3 per cent. alcohol in sea water before fixation.

Trachymedusæ and Acalephæ may be fixed in the usual way in chromic or osmic mixtures. Osmic acid may be added to the sea water containing the animals, which should be removed to fresh water as soon as they begin to turn brown. *Cassiopeia borbonica,* according to Lo Bianco, ought to be treated thus, and then put for two or three days into 5 per cent. solution of bichromate of potash.

See further Lo Bianco, *loc. cit.,* p. 452.

911. Medusæ: Sections.—Paraffin and collodion will afford good sections of some organs, but are certainly not satisfactory as all-round methods for these watery organisms. The Hertwigs (*Nervensystem der Medusen,* 1878, p. 5) imbedded in liver with the aid of glycerin gum, and hardened the objects and the mass in alcohol. Perhaps better results might be obtained by one of the freezing methods given in §§ 195-198.

912. Medusæ: Maceration.—See, especially for the study of the nervous system, § 543. Doubtless in many cases the pyrogallic acid reaction, § 383, would give enhanced differentiation.

913. Siphonophora.—Very difficult, for you have not only to deal with the very great contractility of the zooids, but with the tendency to general *disarticulation* of the swimming bells and prehensile polyps.

The cupric sulphate method of Bedot (*Arch. Sci. phys. et nat.,* xxi, 1889, p. 556) is as follows : A large quantity of

15 to 20 per cent. solution of the salt is suddenly added to the sea water containing the animals. As soon as they are fixed (which happens in a few minutes) a few drops of nitric acid are to be added and mixed in (this is in order to prevent the formation of precipitates), and the whole is left for four to five hours. The specimens are then to be hardened *before* bringing them into alcohol. BEDOT recommends that this be done with strong solution of Flemming, which should be *added* to the solution of sulphate containing the Siphonophore, about two volumes of it being taken for one of the sulphate solution. The whole should be left for at least twenty-four hours. Lastly, a few drops of 25 per cent. alcohol should be added to the fluid with a pipette, being dropped in as far as possible from the colony, which should be disturbed as little as possible; and further alcohol, of gradually increasing strength, should be added so gradually that the strength of 70 per cent. be not attained under fifteen days at least. Ninety per cent. alcohol should be used for definite preservation.

I have tested this method, and find that it *enables one to preserve specimens with all their swimming-bells and polyps in situ*, a result which is not obtained by means of the usual methods.

FRIEDLAENDER (*Biol. Centralbl.*, x, 1890, p. 483) inundates the animals with a mixture of 125 parts cupric sulphate, 125 parts zinc sulphate, and 1000 parts water.

LO BIANCO (*loc. cit.*, p. 454) employs for the majority of Siphonophora a mixture of 10 c.c. of saturated solution of corrosive sublimate with 100 c.c. of 10 per cent. solution of copper sulphate. This is used as in BEDOT's process. *Diphyes*, *Rhizophysa*, and *Physalia*, however, are killed with sublimate solutions; *Velella* with chromo-picric acid, or a mixture of 100 c.c. of sublimate solution with 50 c.c. of 1 per cent. chromic acid; *Porpita* by poisoning with liquid of Kleinenberg.

KOROTNEFF's method has been given, § 15. I have seen *Physophora* very successfully killed by the careful administration of ether.

Preservation, after fixation and washing, is greatly simplified by the use of formaldehyde instead of alcohol (WEBER).

DAVIDOFF (*Anat. Anz.*, xi, 1896, p. 505) fixes in formol.

He gets the animals, in sea water, into a large cylindrical tube (test-tube), plugs its open end with cotton-wool, and stands it up, somewhat sloping, open end downwards, in a vessel half full of 6 to 8 per cent. formol. The formol diffuses up into the tube in about an hour, and kills the animals in extension and with little loss of the swimming-bells ; after which they may either be preserved in the formol itself or be further hardened with other reagents.

914. Ctenophora : Fixation.—The small forms are very easily prepared by means of osmic acid. For the large forms see Lo Bianco, *loc. cit.*, p. 457. He uses his copper sulphate mixture, last §.

Samassa makes sections by the double-imbedding method, § 184 (see *Arch. mik. Anat.*, xl, 1892, p. 157).

Porifera.

915. Spongiæ : Fixation.—The smaller forms can be fairly well fixed by the usual reagents, osmic acid being one of the best. For the larger forms no satisfactory fixing agent has yet been discovered, so far as I can ascertain. The tissues of this group are very watery, very delicate, very friable after hardening, and macerate with the greatest facility. For all but very small specimens absolute alcohol is apparently the best fixing agent. If any watery fluid be preferred, care should at all events be taken to get the sponges into strong alcohol as soon as possible after fixation, on account of the rapidity with which maceration sets in in watery fluids. Fiedler (*Zeit. wiss. Zool.*, xlvii, 1888, p. 87) has been using (for *Spongilla*), besides absolute alcohol, an alcoholic sublimate solution and the liquids of Kleinenberg and Flemming.

Staining.—On account of the great tendency to maceration, I hold that alcoholic stains should be alone employed for staining sponges, and I particularly recommend Mayer's tincture of cochineal, § 252. Von Lendenfeld (*Zeit. wiss. Mik.*, xi, 1894, p. 22) uses aqueous solutions of Congo red and anilin blue for the coloration of collar-cells.

Minchin (*Quart. Journ. Mic. Sci.*, xl, 1898, p. 569) stains spicula sheaths with Freeborn's picro-nigrosin, § 687.

Rousseau (*Ann. Soc. Belg. Mic.*, xxiv, 1899, p. 51) stains in nigrosin, picro-nigrosin, or indulin, or MAYER's picro-magnesia carmine.

For *intra-vitam* staining, see LOISEL, § 221 *ante*, p. 160.

For silvering see § 365.

Sectioning.—Calcareous sponges may be decalcified in alcohol, acidified with hydrochloric or nitric acid, and then imbedded in the usual way. Siliceous sponges may be desilicified by Mayer's method (§ 586).

For ROUSSEAU's methods see § 586. VOSMAER and PEKELHARING decalcify with a solution of picric acid in absolute alcohol (see *Zeit. wiss. Mik.*, xv, 1899, p. 462).

See also Johnstone-Lavis and Vosmaer, § 192.

Preparation of Hard Parts.—Siliceous spicules are easily cleaned for mounting by treating them on a slide with hot concentrated nitric or hydrochloric acid, or solution of potash or soda. The acids mentioned are very efficient, but may attack the silex of some delicate spicules. Thus DEZSÖ found that the small stellate spicules of the cortex of *Tethya lyncurium* are completely dissolved by boiling hydrochloric acid. Potash solution is, therefore, frequently to be preferred, notwithstanding that, in my experience, it does not give such clean preparations.

According to NOLL, *eau de Javelle* is preferable to any of these reagents (see 565).

Embryos and Larvæ.—MAAS (*Zool. Jahrb., Abth. Morph.*, vii, 1894, p. 334) fixes larvæ in liquid of Flemming or Hermann, one to three minutes, and stains with borax-carmine, or with gentian violet and Orange G (Flemming). He also (*Zeit. wiss. Zool.*, lxvii, 1900, p. 218) fixes young Sycones in absolute alcohol and stains with ammonia carmine (spicules *in situ*).

DELAGE (*Arch. Zool. Expér.*, x, 1892, p. 421) fixes larvæ of *Spongilla* that have settled down on cover-glasses for three minutes in absolute alcohol, stains in alcoholic carmine, § 251, and brings through alcohol into oil of bergamot, then either mounts direct in balsam, or detaches the larvæ from the cover and imbeds in paraffin (three minutes).

Protozoa.

916. Introductory.—Since the Protozoa may be considered as free cells, it is evident that the reagents and methods of cytology are in great part applicable to this group. One of the most generally useful of these reagents will be found in the acid solution of *methyl green ;* it is the reagent that allows of the readiest and best demonstration of the presence and form of the nucleus and nucleolus (BALBIANI et HENNEGUY, *Compt. rend. Soc. de Biol.*, 1881, p. 131).

Amongst useful reagents not mentioned in the following sections, I call attention to the weak solutions of alum, potash, and borax, which serve to demonstrate the striations of the cuticle, and the insertions of the cilia of Infusoria.

See also MAGGI, *Tecnica protistologica*, Milano, 1895.

917. Methods for Quieting Infusoria.—See the narcotisation methods, §§ 18 to 22.

According to SCHÜRMAYER (*Jen. Zeit.*, xxiv, 1890, p. 402) nitrate of strychnin in weak solution, 0·01 per cent. or less, gives good results with some forms, amongst which are *Stentor* and *Carchesium*. Antipyrin in concentrated solution (0·1 per cent.), or cocaine of 0·01 per cent., seems only to have given good results as regards the extension of the stalk in stalked forms.

EISMOND (*Zool. Anz.*, xiii, 1890, p. 723) has proposed a mechanical means of slowing the movements of small organisms (small worms and Crustacea as well as Ciliata). He directs that a drop of thick aqueous solution of cherry-tree gum be added to the water containing the organisms (gum arabic and the like, it is stated, will not do). The objects remain fixed in their places, with cilia actively moving, and all vital processes retaining their full activity.

CERTES (*Bull. Soc. Zool. France*, xvi, 1891, p. 93) has found that the method gives excellent results. He has also found that an *intrà vitam* stain may be obtained by adding methyl blue or "violet dahlia, No. 170," to the gum solution.

A similar process has been worked out by JENSEN (after STAHL ; see *Biol. Centralbl.*, xii, 1892, p. 558). A solution of 3 grammes of gelatin in 100 c.c. of ordinary water is made by the aid of heat. This makes a jelly at the normal temperature. It is slightly warmed, and a drop of it is mixed in a watch glass with a drop of water containing the organisms. This plan is said to afford great facilities for the vivisection of Infusoria.

918. Staining *intra vitam.*—See hereon BRANDT (*Verh. physiol. Ges. Berlin*, 1878); CERTES (*Bull. Soc. Zool.*, 25 janv., 1881); and HENNEGUY (*Soc. Philom.*, 12 fév., 1881). See also § 221.

BRANDT recommends a 1 : 3000 solution of Bismarck brown; also (*Biol. Centralb.*, i, 1881, p. 202) "a dilute solution of hæmatoxylin."

CERTES (*op. cit.*, pp. 21, 226, 264, and *Zool. Anz.*, iv, 1881, pp. 208, 287) found that living Infusoria stain in weak solutions of cyanin, Bismarck brown, dahlia, violet 5 B, chrysoidin, nigrosin, methylen blue, malachite green, iodine green, and other tar colours, and hæmatoxylin. The solutions should be made with the liquid that constitutes the natural habitat of the organisms. They should be very weak, that is of strengths varying between 1 : 10,000 and 1 : 100,000. For cyanin, 1 : 500,000 is strong enough.

As to the staining of the *Nucleus*, see PRZESMYCKI, *Biol. Centralb.*, vii, 1897, p. 321; and as to that of the *Granula*, the same author, *Zeit. wiss. Mik.*, xiii, 1896, p. 478. Also LOISEL, § 221.

Examination in a coloured medium in which the organisms do not stain, but show up on a coloured background in a manner that produces somewhat the effect of dark-ground illumination, is sometimes helpful. CERTES (*Bull. Soc. Zool. de France*, xiii, 1888, p. 230) recommends solution of anilin black for this purpose; Infusoria will live in it for weeks. FABRE-DOMERGUE (*Ann. de Microgr.*, ii, 1889, p. 545; *Journ. Roy. Mic. Soc.*, 1889, p. 832) recommends concentrated solution of diphenylamin blue.

919. Demonstration of Cilia (WADDINGTON, *Journ. Roy. Mic. Soc.*, 1883, p. 185).—A drop of solution of tannin, or a trace of alcoholic solution of sulphurous acid, added to the water containing the living organisms.

920. Fixing and Preserving.—For killing by *heat* see § 11.

PFITZNER (*Morph. Jahrb.*, xi, 1885, p. 454) used concentrated solution of picric acid *run in under the cover.*

GEZA ENTZ (*Zool. Anz.*, iv, 1881, p. 575) adds liquid of Kleinenberg to the water containing the organisms *in a watch glass.*

Korschelt (*ibid.*, v, 1882, p. 217) employs in the same way 1 per cent. osmic acid, or, for Amœbæ, 2 per cent. chromic acid.

Lansberg (*ibid.*, p. 336) advises the same reagents, but recommends *bringing the organisms into the fixing liquid with a pipette.*

For fixation with iodine (Kent) or iodine vapour (Overton) see § 88.

For sulphurous acid, § 68.

Cattaneo (*Bollettino Scientifico*, iii and iv; *Journ. Roy. Mic. Soc.*, 1885, p. 538) recommends fixing for a few minutes with ½ per cent. solution of chloride of palladium.

Brass (*Zeit. wiss. Mik.*, 1884, p. 39) employs a mixture of 1 part each of chromic acid, platinum chloride, and acetic acid with 400 to 1000 parts of water.

Certes (*Comptes rend.*, lxxxviii, 1879, p. 433) fixes with 2 per cent. osmic acid, or its vapours (10 to 30 minutes). For details see *previous editions.*

Du Plessis (Vogt et Yung, *Traité, Anat. Comp. Prat.*, p. 92) recommends fixation with 0·2 per cent. solution of corrosive sublimate. Let the preparation *dry up*, and if the organisms have preserved their shape, stain and mount in balsam. This seemingly barbarous procedure is said to give fine preparations when successful.

Fol (*Lehrb.*, p. 102) fixes delicate marine Infusoria (*Tintinnodea*) with the perchloride of iron solution (§ 85), added to the water containing them, and stains with gallic acid as directed, § 384.

Lo Bianco (*loc. cit.*, p. 444) fixes Gregarinæ with picro-sulphuric acid (one hour), Vorticellæ with hot sublimate, Acinetæ with sublimate in sea water, or with osmic acid, Thalassicola with 0·5 per cent. chromic acid (one hour), Acanthometræ and Aulacanthæ with 50 per cent. alcohol or with concentrated sublimate, or by adding a little osmic acid to the water. For Sphærozoa he proceeds as Brandt, § 922.

Zograf fixes Rhizopoda and Infusoria as Rotatoria, § 892, but without narcotisation.

See also the methods of Fabre-Domergue, *Ann. de Microgr.*, ii, 1889, p. 545, and 1890, p. 50; Schewiakoff, *Biblioth. Zool.*, v, 1889, p. 5; *Journ. Roy. Mic. Soc.*, 1889, pp. 832, 833; Zoja, *Boll. Sci. Pavia*, 1892; *Zeit. wiss. Mik.*, ix, 1893, p. 485; Longhi, *Bull. Mus. Zool. Univ. Genova*, 4, 1892; *Zeit. wiss. Mik.*, ix, 1893, p. 483; Lauterborn, *Zeit. wiss. Zool.*, lix, 1895, p. 170; Schaudinn, *ibid.*, p. 193; Balbiani, *Zool. Anz.*, xiii, 1890, p. 133; Karawaiew, *ibid.*, xviii, 1895, p. 286.

921. Sections.—Sections of the larger Protozoa, and amongst them of the larger forms of Infusoria (*Stentor*, *Bursaria*, *Nyctotherus*), may be obtained without much difficulty. The organisms should be strongly fixed, then dehydrated and cleared, and brought into melted paraffin in a small watch glass. After a few minutes therein they are brought on a cataract needle on to a small block of paraffin, and arranged there with a heated needle (p. 93) and sectioned. They may be stained after fixation, or the sections may be stained on the slide, § 200 or 201.

Lauterborn (*loc. cit.* last §) brings the objects through chloroform into paraffin in a small glass tube, and after cooling breaks the tube and so obtains a cylinder of paraffin with the objects ready for cutting.

Hoyer (*Arch. mik. Anat.*, liv, 1899, p. 98) performs all the operations in a glass cylinder (5 cm. long and 7 mm. wide), open at both ends, but having a piece of moist parchment paper tied over one of the openings. It is then not necessary to break the cylinder; by removing the parchment paper the paraffin can be pushed out of it in the shape of a cylinder containing the objects imbedded at one end of it.

See also the watch-glass method, pp. 93 and 94; also Przesmycki, *loc. cit.*, § 918, and Boveri, § 599.

922. Sphærozoa.—Brandt (*Fauna u. Flora Golf. Neapel*, xiii, 1885, p. 7) fixes, according to the species, either with chromic acid of 0·5 per cent. to 1 per cent. (half an hour to an hour), or with a mixture of equal volumes of sea water and 70 per cent. alcohol with a little tincture of iodine for a quarter to half an hour, or with a 5 to 15 per cent. solution of sublimate in sea water.

See also Lo Bianco, § 920.

923. Sporozoa.—Wasielewski (*Sporozoenkunde*, Jena, 1896, p. 153) lays great stress on the study of the living organisms, either in their natural medium, or in normal salt solution, or in a medium composed of 20 parts white of egg, 200 of water, and 1 of common salt. He fixes Gregarinæ and Coccidia with osmic acid, sublimate, or picro-sulphuric acid, and

Myxosporidia with liquid of Flemming. He stains Gregarinæ with safranin, picro-carmine, etc., besides employing gold chloride, silver nitrate, acetic acid, ammonia, etc., and Myxosporidia with safranin or gentian and eosin.

SCHAUDINN (*Zool. Jahrb., Abth. Anat.*, xiii, 1900, p. 197) finds the best fixative a mixture of 2 parts of saturated aqueous sublimate and 1 of absolute alcohol, with, if desired, a trace of acetic acid.

PIANESE (*Arch. Parasit.*, ii, 1899, p. 412) fixes liver infected with Coccidia for thirty-six hours in a mixture of 20 c.c. of 10 per cent. aqueous solution of chloride of cobalt, 5 c.c. of 2 per cent. osmic acid, and a drop of formic acid.

924. Hæmatozoa.—GRASSI (*Att. Accad. Lincei*, iii, 1900, p. 357) demonstrates the Malaria-parasites in the intestine, body cavity, and salivary glands of *Anopheles* by treating them with normal salt solution containing 2 per cent. of formol (pure formol produces swellings), or in a mixture of 1·5 grm. of salt and 250 c.c. of water with the white of an egg. He fixes with sublimate, makes paraffin sections, and stains with hæmalum or iron hæmatoxylin. He stains the Sporozoïtes by making cover-glass preparations which are allowed to dry, put for twenty-five minutes into absolute alcohol, and stained by the process of ROMANOWSKY, § 719.

LAVERAN (*C. R. Soc. Biol.*, li, 1899, p. 249) stains the nuclei of endoglobular parasites with "bleu BORREL," which is made as follows: A solution of nitrate of silver is precipitated by caustic soda, the precipitate of silver oxide is carefully washed and added, with long-continued agitation, to a concentrated solution of methylen blue, which is then allowed to stand for several days and decanted. Cover-glass films of blood are made, dried, and fixed for an hour in absolute alcohol. They are stained for twelve to twenty-four hours in a freshly prepared mixture of 1 part of "bleu Borrel," 5 of 0·1 per cent. aqueous solution of eosin and 4 of water, washed with water, put for two minutes into 5 per cent. solution of tannin, washed, dried, and mounted in balsam.

For *Herpetomonas* see next §.

For recent modifications of the ROMANOWSKY-ZETTNOW stain, see MARINO in *Ann. Inst. Pasteur*, xviii, 1904, p. 761, or *Zeit. wiss. Mik.*, xxi, 1905, p. 491, and GIEMSA, *ibid.*, p. 522, or *Centralb. Bakt.*, 1904, p. 308.

925. Flagellata.—LAUTERBORN (*Zeit. wiss. Zool.*, lix, 1895, p. 170) fixes *Ceratium* for about ten minutes in liquid of Flemming, puts into alcohol for twenty-four hours, brings back into water, bleaches if necessary with hydrogen peroxide, and stains with picrocarmine or Delafield's hæmatoxylin. He also imbeds in paraffin, § 921, and stains sections with iron hæmatoxylin.

WASIELEWSKI and SENN (*Zeit. Hyg.*, xxxiii, 1900, p. 451) study *Herpetomonas* by making dry cover-glass films of blood, fixing them by heat or absolute alcohol, and staining by the method of ROMANOWSKY, § 719.

ZACHARIAS (*Zool. Anz.*, xxii, 1899, p. 72) fixes *Uroglena*, etc., with a mixture of 2 vols. saturated aqueous solution of boracic acid and 3 of saturated sublimate.

926. Stains for Flagella.—The process of ROMANOWSKY and ZETTNOW, § 719, will give a red stain of the flagella of some forms.

The method of LÖFFLER has run through several forms (*Centralbl. Bakteriol.*, vi, 1889, p. 209; vii, 1890, p. 625; *Zeit. wiss. Mik.*, vi, 1889, p. 359; vii, 3, 1890, p. 368; *Journ. Roy. Mic. Soc.*, 1889, p. 711; 1890, p. 678), of which that given here is the latest. To 10 c.c. of a 20 per cent. solution of tannin are added 5 c.c. of cold saturated solution of ferrous sulphate and 1 c.c. of (either aqueous or alcoholic) solution of fuchsin, methyl violet, or "Wollschwarz."* Cover-glass preparations are made and fixed in a flame in the usual way, special care being taken not to over-heat. Whilst still warm the preparation is treated with mordant (*i. e.* the above-described mixture), and is heated for half a minute, until the liquid begins to vaporise, after which it is washed in distilled water and then in alcohol. It is then

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[*] The mixture will require *for some forms* the addition of a few drops of 1 per cent. solution of caustic soda; *e. g.* for typhoid bacilli, 1 c.c.; for *Bacillus subtilis*, 28 to 30 drops; for bacilli of malignant œdema, 36 to 37 drops. Some other forms will require besides the addition of a trace of sulphuric acid to the soda solution—so for cholera bacteria, half a drop to 1 drop; for *Spirillum rubrum*, 9 drops.

treated in a similar manner with the stain, which consists of a saturated solution of fuchsin in anilin water (p. 203), the solution being preferably neutralised to the point of precipitation by cautious addition of 0·1 per cent. soda solution.

BUNGE (*Journ. Roy. Mic. Soc.*, 1894, p. 640; *Zeit. wiss. Mik.*, xiii, 1896, p. 96) makes the mordant by mixing three parts of the tannin solution with 1 of *liquor ferri sesquichlorati* diluted twentyfold with water, and lets the mixture ripen for some days exposed to the air, or (*Journ.*, 1895, pp. 129, 248) adds to it a few drops of hydrogen peroxide, until it becomes red-brown, when it is shaken up and filtered on to the cover-glass and allowed to act for a minute. The cover-glass is then mopped up and dried, and stained with carbol-gentian.

KOERNER and FISCHER (quoted from *Encycl. mik. Techn.*, p. 428) make the mordant with 2 parts of tannin, 20 of water, 4 of ferrous sulphate solution of 1 : 2 strength, and 1 of saturated alcoholic solution of fuchsin. Warm, let it act for a minute, rinse and stain with anilin-water-fuchsin, or carbol-fuchsin.

Similarly ELLIS (*Centralb. Bakt.*, xxi, 1903, p. 241; *Journ. Roy. Mic. Soc.*, 1904, p. 249), but staining with Säureviolett, 1 part to 75 of alcohol and 75 of water.

PEPPLER (*Centralb. Bakt.*, xxix, 1901, p. 376; *Zeit. wiss. Mik.*, xviii, 1901, p. 222) dissolves 20 parts of tannin in 80 of water, and adds gradually 15 parts of 2·5 per cent. chromic acid, allows to stand for a few days at a temperature of not less than 18° C., filters and preserves in closed flasks. This mordant will keep for months. Mordant for about five minutes and stain as above.

ROSSI (*Arch. per le Sc. med.*, xxiv, 1900, p. 297; *Zeit. wiss. Mik.*, xviii, 1901, p. 226) gives the following as very simple and easy. The mordant is a solution of 25 grms. of tannic acid in 100 of caustic potash of 0·1 per cent., and will keep indefinitely. The stain is Ziehl's carbol-fuchsin, § 301. Cover-glasses are prepared with a drop of culture, dried, and treated with 1 drop of the mordant and at the same time 4 to 5 of the stain, allowed to remain for 15 to 20 minutes, washed, and mounted. A more complicated modification of this is described in *Centralb. Bakt.*, xxxiii, 1903, p. 572 (*Zeit. wiss. Mik.*, xix, 1903, p. 517).

Gemelli (*Centralb.*, xxxiii, 1903, p. 316; *Zeit. wiss. Mik.*, xix, 1903, p. 516) mordants for 10 to 20 minutes in 0·025 per cent. permanganate of potash, rinses and stains for 15 to 30 minutes in a mixture of 20 parts 0·75 per cent. aqueous solution of calcium chloride and 1 part of 1 per cent. neutral red solution.

A method of Pitfield is described by Kendall, *Journ. app. Mic.*, v, 1902, p. 1836 (*Journ. Roy. Mic. Soc.*, 1902, p. 502). The mordant consists of 10 parts of 10 per cent. tannin solution, 5 parts of saturated sublimate solution, 5 of saturated solution of alum, and 5 of carbol fuchsin. Mordant for a minute with heat, and stain with a mixture of 2 parts saturated aqueous solution of gentian violet with 10 of saturated solution of alum.

Van Ermengem (*Journ.*, 1894, p. 405) fixes for a few minutes with a mixture of 1 part 2 per cent. osmic acid, and 2 parts 10 to 25 per cent. solution of tannin, washes, treats with 0·25 to 0·5 per cent. solution of nitrate of silver, then for a few seconds with a mixture of 5 parts gallic acid, 3 of tannin, 10 of acetate of soda, and 350 of water, then puts back again into the silver for a short time, then washes and mounts.

See also Stephens, *ibid.*, 1898, p. 685, and Gordon, *ibid.*, 1899, p. 235, and the methods of Trenkmann (*Centralbl.*, vi, 1889, p. 433; *Zeit. wiss. Mik.*, vii, 1890, p. 79); Brown (*Journ. Roy. Mic. Soc.*, 1893, p. 268); Julien (*ibid.*, 1894, p. 403); Sclavo (*Zeit. wiss. Mik.*, xiii, 1896, p. 96); Hessert (*ibid.*, p. 96); Muir (*Journ. Roy. Mic. Soc.*, 1899, p. 235); McCrorie (*ibid.*, 1897, p. 251; he stains for two minutes in a mixture of equal parts of concentrated solution of night-blue, 10 per cent. solution of alum, and 10 per cent. solution of tannic acid); Zettnow (*ibid.*, 1899, pp. 662, 664); Morton (*ibid.*, 1900, p. 131); Welcke (*ibid.*, p. 132).

APPENDIX.

927. Table for diluting Alcohol (after Gay-Lussac).—To use this table, find in the upper horizontal row of figures the percentage of the alcohol that it is desired to dilute, and in the vertical row to the left the percentage of the alcohol it is desired to arrive at. Then follow out the vertical and horizontal rows headed respectively by these figures, and the figure printed at the point of intersection of the two rows will show how many volumes of water must be taken to reduce *one hundred volumes* of the original alcohol to the

Weaker grade required.	Original Grade.								
	90 p. 100.	**85** p. 100.	**80** p. 100.	**75** p. 100.	**70** p. 100.	**65** p. 100.	**60** p. 100.	**55** p. 100.	**50** p. 100.
p. 100. **85**	6·56								
80	13·79	6·83							
75	21·89	14·48	7·20						
70	31·05	23·14	15·35	7·64					
65	41·53	33·03	24·66	16·37	8·15				
60	53·65	44·48	35·44	26·47	17·58	8·76			
55	67·87	57·90	48·07	38·32	28·63	19·02	9·47		
50	84·71	73·90	63·04	52·43	41·73	31·25	20·47	10·35	
45	105·34	93·30	81·38	69·54	57·78	46·09	34·46	22·90	11·41
40	130·80	117·34	104·01	90·76	77·58	64·48	51·43	38·46	25·55
35	163·28	148·01	132·88	117·82	102·84	87·93	73·08	58·31	43·59
30	206·22	188·57	171·05	153·61	136·04	118·94	101·71	84·54	67·45

required grade. Thus, if it be required to manufacture some 70 per cent. alcohol, starting with 90 per cent., we find the figure 90 in the upper column, the figure 70 in the vertical column, and at the point of intersection we read 31·05, showing that a fraction more than 31 volumes of water must be added to 100 volumes of 90 per cent. alcohol. Or similarly, if we wish as before to make 70 per cent. alcohol, but start with an alcohol of 85 per cent., we find that 23·14 volumes of water must be employed.

928. Chemicals, Stains, and Apparatus.—Addresses from which it is recommended that these be obtained are given in § 224.

929. Cleaning Slides and Covers.—*New* ones should first be soaked in one of the following liquids: strong sulphuric, hydrochloric or nitric acid, or *aqua regia,* or a mixture of an ounce each of sulphuric acid and bichromate of potash with from 8 to 12 ounces of water, then washed first with water and lastly with alcohol, and dried with a clean cloth.

For *used* ones, if a balsam mount, warm, push the cover into a vessel with xylol or other solvent of the mount, and put the slide into another vessel with the same, leave for a few days, and then put into strong alcohol. If this is not sufficient, treat as for new ones. Some persons boil in lysol, which I do not find efficacious.

For the final treatment, see p. 140.

930. Gum for Labels.—Labels stuck on glass often strip off. This may be avoided (MARPMANN, *Zeit. Angew. Mik.*, ii, 1896, p. 151; *Journ. Roy. Mic. Soc.*, 1897, p. 84) by means of the following adhesive: 120 grammes of gum arabic are dissolved in a quarter of a litre of water, and 30 grammes of gum tragacanth in a similar quantity. After a few hours the tragacanth solution is shaken until it froths, and mixed with the gum arabic solution. Strain through linen and add 150 grammes of glycerin previously mixed with 2½ grammes of oil of thyme.

PEIRCE (*Journ. app. Mic.*, ii, 1899, p. 627; *Journ. Roy. Mic. Soc.*, 1900, p. 404) finds that if the end of the slide be painted with a thin solution of balsam, it may be written on with ink when dry, and the record preserved by a second coat painted over it.

For other receipts see *previous editions.*

931. New Water-bath: Addendum to § 147.—The Cambridge Scientific Instrument Co. have brought out a new water-bath which can be heated by a petroleum lamp, and maintains a constant temperature to within 1° C.; see figure in *Journ. Roy. Mic. Soc.*, 1905, p. 114.

932. Vacuum Imbedding Stove: Addendum to § 148.—An apparatus for this purpose is described by FUHRMANN in *Zeit. wiss. Mik.*, xxi, 1905, p. 462.

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